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# CHEMICAL METHODS IN CLINICAL MEDICINE

THEIR APPLICATION AND INTERPRETATION  
WITH THE TECHNIQUE OF THE SIMPLE TESTS

By

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manuscript, and to both him and Mr G Discombe for several helpful criticisms. I thank my assistants, Mr R Hudson and Mr C Wyatt, for help in descriptions of technique and in reading proofs.

The Lovibond comparator and discs of coloured glass standards have been valuable in the routine determinations of proteins by the biuret method, of carboxyhæmoglobin in blood, etc., and I am indebted to Messrs The Tintometer Ltd, and especially to Mr G S Fawcett, for the trouble taken in matching the chemical standards accurately.

A few paragraphs in the book have been published previously, and acknowledgements are tendered to the *Lancet*, *British Medical Journal*, *Archives of Diseases in Childhood*, *Clinical Journal Medical Press and Circular*, and *St Bartholomew's Hospital Report*.

For the use of figures thanks are given to Messrs The Tintometer Ltd (Figs 7 and 52), Drs Peters and Van Slyke (Fig 23), Drs Gamble, Ross and Tisdall (Fig 40), Messrs J Swift and Son, Ltd (Fig 45), Messrs Down Bros Ltd (Fig 60), Messrs Bellingham and Stanley, Ltd (Fig 62), Messrs Gullenkamp & Co Ltd (Fig 66), Drs Hawk and Bergeim (Fig 72), Drs Bennett and Dodds (Fig 74) and Mr Kendrick (Fig 82).

G A H

LONDON

# FROM THE PREFACE TO THE FIRST EDITION

"Which book gives the subjects dealt with in your classes?" "What is the meaning of these analytical results?" "How do you estimate such and such a substance?" These questions are commonly asked by the student, the practitioner and the laboratory worker respectively. The author has attempted to provide answers to all these and to similar questions. The book is written essentially from the clinical point of view. The application and interpretation of the more commonly employed methods alone are described, but it is hoped that by consulting the references to books and to articles in journals, the reader will be able to cover *the greater portion of the field*.

There has been the usual difficulty in deciding how much technical matter shall be included. The author has drawn the line so as to embrace practically everything which is regarded as a routine analysis in his own department. Here and there this dividing line has been overstepped so as to include personal observations. It will be noted that the calculations are given very fully—some readers may consider in absurd detail. This has been done purposely, because, in the author's experience, more mistakes are made in the calculations than in the actual technique by the average student and technical assistant, and also because, in his opinion, many writers give calculations too briefly.

Both the "old" and the newer parts of the subject have been discussed, in an attempt to include the essentials of the whole of clinical chemical pathology in one book. Considerable stress has been laid on qualitative urinary tests, because the author finds that a large portion of his work is made necessary by a lack of knowledge of this essential branch on the part of those working in the wards. Indeed, he feels that there is a grave risk of the student to-day suffering as a result of the tendency, on the one hand, the clinical worker to refer urine tests to the laboratory, and, on the other hand, for the laboratory worker to be so interested in the newer methods of blood analysis, etc., that he is apt to give insufficient attention to examination of the urine. An attempt has been made to present this "older" part of the subject in a way which differs from the usual arrangement, and in a way in which it seems to the author that the problems are tackled in practice. The greater part of the volume deals with the subject as a natural extension of the routine chemical examination of the urine.

Starting with this, as outlined in Chapter II, a course of practical classes and lectures in chemical pathology may easily be developed

by the teacher in accordance with laboratory facilities and the status of his students. In fact, the book is largely based on the writer's own scheme of lectures and practical classes.

A full index has been provided, which includes an index of diseases. It is hoped that this will enable the reader to see at a glance what chemical tests may be performed in a given disease, and, by reference to the text, to discover the likelihood of their value. . . .

G. A. HARRISON.

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# CHEMICAL METHODS IN CLINICAL MEDICINE

## CHAPTER I

### INTRODUCTION

#### THE FIELD OF CHEMICAL PATHOLOGY

CHEMICAL pathology in the wide sense is a huge subject, and may be defined as the application of chemistry, of physiological chemistry, and of other preliminary sciences with a chemical flavour, to medical problems. The ideal chemical pathologist must have not only a very wide training, but also a highly specialised training both in the theory and technique of several subjects. Chemical pathology, therefore, is biochemistry applied to medicine. In the narrower sense the chemical pathologist is a man who does certain chemical tests on material obtained from patients at the request of his clinical colleagues. He is simply a "medical analyst". In the writer's opinion, this attitude to chemical pathology should be strongly discouraged, since it does not make for the best work, either in diagnosing and treating patients or in advancing knowledge of disease. The better type of chemical pathologist knows the value and limitations of the tests from the technical side, and from his experience, shared with not one but many clinical colleagues in all branches, he in time learns much of the value and limitations of his tests clinically. The sound clinician is an expert on the clinical side, and also has a general knowledge of laboratory tests, but particular knowledge of their value and limitations in his own branch. The combination of the two types of men makes for the best work, but it can only be rarely that the two are combined in one man, so large have the subjects become.

When teaching chemical pathology it is not uncommon to hear the student complain that he has done all this before in chemical physiology. That complaint is to a certain extent justified since much of chemical pathology is the application of physiological chemical tests to pathological material. But whereas in chemical physiology the student learns a series of tests for one or another substance, in chemical pathology he has to select and apply only those tests which can be relied upon to give him information in more complicated fluids, more complicated in the sense that owing to disease there may be not one but several abnormalities. For example, in chemical physiology the student learns to test the urine for glucose, or for lactose, or for pentose, and so on. In chemical

pathology he has to apply this knowledge, or a selected part of it, to identify the substance or mixture of substances responsible for the reducing action he observes the urine to give

It is obviously not only unnecessary, but impossible to cover the ground which is supposedly covered in the student's preliminary studies, but the student is bound to forget something, and not to have the opportunity of learning everything. The writer, therefore, makes no apologies for including in this and subsequent chapters certain chemical and technical points which in his experience cause difficulty to students and technical assistants

Naturally in medicine preference is often given to the simpler tests. Not uncommonly only gross variations from the normal are of pathological significance. When interpreting the results of analyses, it is important to know the normal range (and not merely the "average normal"), and also the percentage accuracy of the technique employed. It is often fair to state that such and such a method with an error of  $\pm x$  per cent is sufficiently accurate for clinical purposes, but strong objection is taken to the phrase "good enough for clinical work". The chemical pathologist should be at least as careful in his technique as his scientific colleagues. He may select a method which is relatively simple, and has a probable error of, say,  $\pm 10$  per cent, in preference to a more elaborate technique which has a probable error of, say,  $\pm 2$  per cent, because, and only because, he knows that the better method will not yield results of any more significance clinically. For example, in the urea concentration test (see Chapter V), the hypobromite method of estimating urea in urine is selected in preference to the more accurate urease method, because it is much simpler, and only gross variations in the urea concentration are of significance clinically.

## SELECTION OF BOOKS

The reader already possesses the particular book on chemical physiology used in his school, together with a text book of physiology for reference. Of the former, Hawk and Bergeim's *Practical Physiological Chemistry* is one of the most comprehensive, and gives numerous references to the literature. Another valuable book is Mathew's *Physiological Chemistry*, which gives a fuller discussion of various theories. A very wide field is covered by Mandel's translation of Hammarsten and Hedin's *Text book of Physiological Chemistry*.

On entering upon our clinical studies most of us find Hutchison and Hunter's *Clinical Methods* invaluable, and this includes several of the chemical tests. There are innumerable books on Clinical Diagnosis or Clinical Pathological Methods from which to make a selection, but everyone really interested in the chemical side of general pathology should possess a copy of Wells' *Chemical Pathology*. Neubauer Huppert's *Analyse des Harns* is a mine of information, but has not been brought up to date. Peters and Van Slyke's *Quantitative Clinical Chemistry* (Vol I, Interpretations, Vol II,



Methods) is the standard work, and is of great value for reference, though chemical tests in jaundice and gastric analysis receive little attention

For other books and particularly those on special subjects, the reader is referred to the lists at the beginning of the different chapters

### DEFINITION OF CERTAIN TERMS

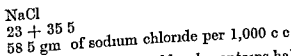
The term "normal" may cause confusion. It is used in no less than three different senses with regard to salts

(i) A "normal salt" in chemistry is one which contains no replaceable hydrogen or hydroxyl ions or basic oxygen, as opposed to an *acid* salt which contains replaceable H or a *basic* salt which contains replaceable OH or basic oxygen, e.g.,

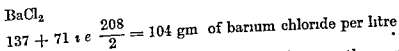
<i>Normal</i>	<i>Acidic</i>
$\text{Na}_3\text{PO}_4$ Tri sodium phosphate	$\text{NaH}_2\text{PO}_4$ Sodium dihydrogen phosphate
COOK	COOK
COOK "neutral" potassium oxalate	acid potassium oxalate
	COOH
	<i>Basic</i>
	$\text{BiOCl}$ from $\text{Bi}_2\text{O}_3$
	$\text{Fe}(\text{OH})\text{SO}_4$ from $\text{Fe}(\text{OH})_3$

Solutions of "normal" salts are not necessarily neutral in reaction, e.g.,  $\text{Na}_2\text{CO}_3$  which is alkaline, and  $\text{FeCl}_3$  which is acid, though the term "neutral" is not uncommonly used instead of "normal" (e.g., "neutral potassium oxalate" instead of "normal potassium oxalate")

(ii) A "normal solution" of a salt is the "equivalent per litre" solution. In the case of substances whose molecular weights in grammes contain 1 gm of replaceable hydrogen, or 17 gm of replaceable hydroxyl, or 35.5 gm of chlorine, and so on, the normal solution is the same as the "molar" solution (molecular weight in grammes in 1,000 c.c.), but the molar solutions of some substances contain multiples of the equivalent weights. Thus, a normal solution of sodium chloride is the molecular weight in grammes, or 58.5 gm per litre, or 5.85 per cent —



But a normal solution of barium chloride contains half the molecular weight, or 104 gm of anhydrous barium chloride in a litre of solution —



In other words, with a knowledge of valencies, the normal

solutions of salts can easily be calculated. In the above example  $\text{BaCl}_2$  has been calculated as the anhydrous salt. The barium chloride in general use is the hydrated salt. It is essential to know whether a salt is hydrated or anhydrous. Very frequently the makers do not state whether a salt is hydrated or not, and this is often the cause of error, e.g.,

$$\text{BaCl}_2 \cdot 2\text{H}_2\text{O} \text{ (crystalline barium chloride)}$$

$$208 + 36 \times 2 = \frac{244}{2} = 122 \text{ gm per litre}$$

Authors, too, not infrequently neglect to mention the water of crystallisation of the salts employed. Thus the sodium tungstate used in Folin's method of precipitating proteins contains two molecules of water, and the zinc sulphate of Hagedorn and Jensen's blood sugar method is  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ .

(iii) The "normal" saline used in bacteriology and physiology, etc., is 0.85 per cent sodium chloride, whereas a normal solution of  $\text{NaCl}$  is 5.85 per cent (see above). This third use of the term "normal" may unfortunately be confused with the other two technical meanings. Better names are "physiological" saline, or "isotonic" saline (because it has the same osmotic pressure as human blood).

The Terms "Weak" and "Strong," and "Diluted" and "Concentrated"—Strictly, the term "weak" or "strong" acid or base should be used in reference to the degree of ionisation, and not in reference to the degree of dilution. Thus  $\text{HCl}$  is a strong acid because it is almost completely ionised in solution. Acetic acid is a weak acid because it is only slightly ionised. When a solution has been diluted, it should not be referred to as a "weak" solution. Similarly, a concentrated solution should not be termed "strong," e.g., ammonia solution with a S.G. of 0.88 is concentrated ammonia, and not strong ammonia.

## PREPARATION OF STANDARD SOLUTIONS

Some examples are given in the Appendix, but the definition and general principles of the preparation of standard ("normal") solutions as used in volumetric analysis are as follows—

*A normal solution is such that 1 litre yields 1 gm of replaceable hydrogen, or 17 gm of replaceable hydroxyl, or 35.5 gm of chlorine or 8 gm of replaceable oxygen, and so on.* It is an 'equivalent per litre' solution. If this definition is remembered there will be no difficulty.

*A normal solution of an acid can be looked at in another way.* It is equal to the molecular weight in grammes over the basicity of the acid per litre. Sulphuric acid is a dibasic acid, i.e., it contains two replaceable hydrogen atoms.

Normal = Equivalent per litre

Normal =  $\frac{\text{Molecular weight}}{\text{Basicity of acid}}$  in grammes per litre

Thus, instead of 98 gm of sulphuric acid,  $\frac{98}{2} = 49$  gm of sulphuric acid per litre will be required. In the same way, for phosphoric acid,  $H_3PO_4$ , which contains three replaceable hydrogens, a normal solution will contain  $\frac{98}{3}$ , or 32.67 gm per litre, and so on.

The normal solution of a base contains 17 gm of replaceable hydroxyl, or 8 gm of basic oxygen per 1,000 c.c. It may be expressed as the molecular weight in grammes over the acidity of the base per litre, *e.g.*, with caustic soda —

$$\frac{NaOH}{40} \times e = \frac{40}{1} \text{ gm per litre}$$

On the other hand, for calcium hydroxide, containing two replaceable hydroxyls, 37 gm per litre would be needed —

$$\frac{Ca(OH)_2}{74} \times e = \frac{74}{2} = 37 \text{ gm per litre,}$$

though, in fact, calcium hydroxide is not soluble in water to this extent.

Very often, the substances cannot be obtained absolutely pure. Thus HCl always contains water, and in varying amounts, as can easily be shown by taking the specific gravity. Often it is impossible to weigh out a substance that is going to be made up into a standard solution. Thus some substances, *e.g.*, sulphuric acid, may take up water during weighing. Sodium hydroxide absorbs  $CO_2$  from the air. For these reasons and others, the following indirect method has often to be used. First, a solution of approximately the correct concentration has to be made. For this purpose the following table will be useful —

*Approximate Normality of Acids and Bases*

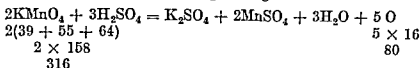
Substance	Approx strength of original substance	c c substance per litre to make approx ~		
		10 N $\frac{N}{1}$	0.1 N $\frac{N}{10}$	0.01 N $\frac{N}{100}$
<i>Acids</i>				
Acetic	17 N	60 c c	6 c c	0.6 c c
HCl	10 N	100 "	10 "	1.0 "
HNO <sub>3</sub>	16 N	63 "	6.3 "	0.7 "
H <sub>2</sub> SO <sub>4</sub>	36 N	28 "	3 "	0.3 "
<i>Bases</i>				
NH <sub>4</sub> OH	20 N	50 c c	5 c c	0.5 c c
KOH	—	58 gm	5.8 gm	0.6 gm
NaOH	—	42 ,	4.2 ,	0.42 ,

Then the approximate solution must be estimated accurately against a standard acid or base, etc., and then diluted to the required amount. As an example, suppose 10 c c of an approximately  $N/10$  solution of  $HCl$  require 11 c c of exactly 0.1  $N$  soda, then each 10 c c of the approximate solution of  $HCl$  must be made up to 11 c c, with water.

A normal solution of a salt has already been described.

Normal solutions of oxidising agents may be defined as solutions which yield 8 gm of available oxygen or its equivalent per 1,000 c c.

Take as an example potassium permanganate in acid solution —



From the above equation it is seen that 316 gm of  $KMnO_4$  in acid solution yield 80 gm of oxygen, therefore, from the above definition 31.6 gm of  $KMnO_4$  yield 8 gm of oxygen and would be contained in 1,000 c c of a normal solution.

## NOTES ON CERTAIN APPARATUS

### Pipettes

Pipettes may be calibrated in one of three ways (i) "to deliver" by touching off against the side of the vessel (for fifteen seconds) when the pipette is filled, and again when it is emptied, (ii) "to blow out," the pipette is filled to the mark and wiped clean externally, drained into the vessel, and finally blown through once whilst drawing it upwards and touching off, (iii) "to contain." The pipette is filled to the mark, superfluous fluid on the outside or at the end being removed with filter paper, lint, etc. The contents are then delivered into the diluting fluid, which is then sucked up and down repeatedly, the pipette being finally blown as dry as possible.

In (i) and (ii) calibration is usually effected by weighing water, in (iii) by weighing mercury. For examples, see Appendix. When checking the calibration, pipettes that are accurate  $\pm 1$  per cent may be passed for all ordinary purposes. Sometimes pipettes as obtained from the makers are as much as  $\pm 5$  per cent out. For very accurate work each pipette should be calibrated by the operator himself with the fluid he is going to use, but this is unnecessary for clinical work. Pipettes should be clean and dry. Mucus from saliva, grease, etc. in time may introduce appreciable errors. Routine cleaning and drying may be carried out by rinsing through in order with tap water, distilled water and acetone. The pipette is then placed in a suitable stand tip uppermost, and air drawn through with the aid of a suction pump until it is quite dry.

(Fig 1) When a pipette is dirty, as shown by drops of fluid adhering to its walls when drained, or by bubbles of air refusing to move when it is filled, it should be washed through with tap water and placed overnight in dichromate cleaning fluid (see Appendix).

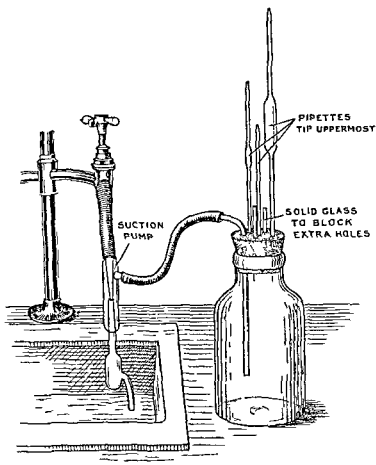


FIG 1 Apparatus for drying pipettes

Next day it is rinsed in turn with tap water, distilled water, and acetone, and dried as previously described.

### Microscope

The care of the microscope is outside the province of this book, but a note on focussing the condenser is given below because the writer has found that very few students know how to do it

Place a blood film, a mounted urinary deposit or other object on the stage and focus it accurately, using the low power (*e.g.*,  $\frac{2}{3}$  in.) Thereafter do not alter the setting of the microscope above the stage. Rack up the Abbé condenser until it almost touches the underside of the microscopical slide. Tilt the reflecting mirror until a chimney pot or some other convenient distant object also comes into view on looking down the microscope. Raise or lower the condenser till the distant object also is sharply in focus. The condenser is now correctly focussed and should not be moved again. Tilt the mirror to get rid of the image of the distant object (chimney pot, etc.) Open or close the diaphragm to alter the intensity of the

**illumination** The same procedure may be employed for the higher power (*e.g.*,  $\frac{1}{2}$  in). It is perhaps a refinement of technique but makes a considerable difference to the definition of small objects and reduces eye strain during prolonged examinations.

A simple pointer placed in the eyepiece is illustrated in Fig. 2. This is useful to the teacher for demonstration, and to the student in asking his instructor what is the nature of a particular object.

A thick ring is cut from a piece of rubber tubing of suitable size, in the rubber is inserted a pointer made by filing to a sharp point a piece of stilette wire, or by utilising the terminal  $\frac{1}{2}$  in. of a fine needle. The rubber ring is placed on the diaphragm in the

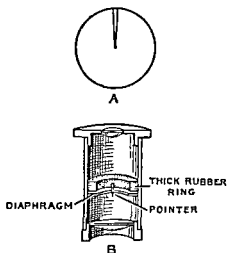


FIG. 2. Pointer for eyepiece of microscope.

A. As viewed down microscope.

B. As seen in section of eye piece.

eyepiece and the pointer is adjusted so that its point lies in the plane of the diaphragm, and is sharply in focus when using the microscope.

### Polarising Microscope

It is valuable to possess a polariser and analyser which can be attached to the microscope, or, better, a petrological microscope with rotating stage, polariser and analyser. The apparatus is useful in the identification of starch granules (p. 58), of doubly refracting droplets in casts from myelin kidneys (p. 46), in examining crystals and fatty deposits in sections (*e.g.*, in xanthomatosis), and so on. In general, the great majority of organic crystals are doubly refracting or "anisotropic". Indeed, all crystals organic and inorganic, are anisotropic if they do not belong to the cubic system.

### Electric Centrifuge

A description of this is unnecessary, but it is important to see that the buckets with their contents are properly counterpoised, using a balance for the purpose (Fig. 3).

The electric current should be switched on, and then the resistance should be slowly taken out, pausing at intervals to allow the machine to gather speed. When stopping the machine, the resistance should be put in first, and the current switched off last, to prevent the next person who uses the machine from inadvertently switching on the current with the resistance out of circuit, and thereby throwing great strain on the apparatus.

Periodically (*e.g.*, once a week or once a fortnight) the machine

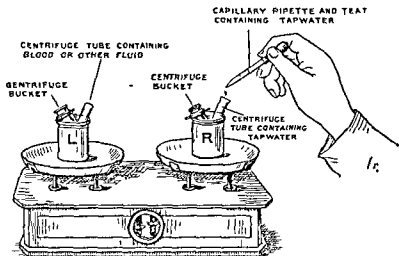


FIG 3. Balancing centrifuge tubes

should be lubricated according to the maker's instructions. If no instructions are supplied, write to the makers.

### Direct Vision Spectroscope

This is invaluable in chemical pathology. For instructions for use, see p. 208.

### Colorimeter

This is an apparatus for comparing the intensity of the colour of two solutions, one of which is termed the "standard" and the other the "unknown". The "standard" contains a known amount of a given substance in suitable solution and is treated with a particular reagent to produce a coloured compound. The "unknown" contains an unknown amount of the same substance in solution and is treated in exactly the same way as the "standard."

A description of the various types of colorimeters is given in the standard text-books. It is essential to remember certain general principles. Unless it has been shown by experiment that the depth

of colour is proportional over a wide range to the concentration of substance present, the standard and unknown must be of approximately the same concentration, they should not differ by more than 20 per cent. For this reason two or more standards of different concentrations are frequently prepared simultaneously with the unknown, and that standard which approximates most closely in colour to the unknown is selected for the final comparison. Secondly, in developing colours in complicated mixtures such as blood or urine, it not uncommonly happens that coloured compounds are obtained from substances other than the substance under investigation, and though the conditions are arranged so that these extra colours cause the minimum of interference they do at times introduce various tints and modifications of the main colour, so that colour comparison is inaccurate or impossible. Thirdly, both standard and unknown should be absolutely clear. Occasionally a precipitate is formed in the unknown which may make colorimetric comparison impossible or inaccurate. Difficulties due to variation in tint, or to opacities, are particularly likely to be encountered in pathological work. The standard is usually a solution of the pure substance to be estimated, in water or some other solvent (volatile solvents are obviously undesirable, but sometimes have to be used). The unknown is a solution of the same substance in some complicated solution such as urine, urine filtrates, protein free filtrates of blood, and so on. Theoretically the standard should be prepared by dissolving  $x$  in the unknown deprived of its content of  $x$ , but this is obviously generally impossible. "Artificial standards" are sometimes used. These are of two main types: (i) a solution of another chemical which has the same colour as the solution of the substance to be estimated (cf. standard in Van den Bergh's test, Chapter XII), (ii) glass discs of appropriate colours. Rosenheim and Schuster have developed a colorimeter on this principle (*Biochem J*, 1927, 21, 1329). The above points are important in indicating the limitations of colorimetry. In spite of these limitations, however, colorimetric methods are valuable, and particularly in chemical pathology, on account of their relative simplicity and the speed with which repeated estimations of the same substance can be performed. Moreover, colorimetric methods are often applicable to much smaller quantities of fluid (e.g., blood) than the more orthodox volumetric or gravimetric methods. Indeed, in medicine it may be a question of a colorimetric method or none at all.

The following are the essential practical instructions (Kober type of apparatus) —

(i) Clean and dry both cups and plungers

(ii) Adjust (if necessary) the zero readings. Rack up each cup till it touches the bottom of the plunger, and see that 0 corresponds to 0 on each side. If not, reset the scale. In the Kober type make sure that the base of the cup sits properly on the base of the holder.

(iii) Fill both cups with the standard solution. Set each at, say, 20 mm, and adjust the mirrors till the illumination is even.



In the newer types of colorimeter with an electric bulb in the base as direct light source, the position of the bulb is adjusted till the illumination of the two half fields is equal. A set screw is provided which is loosened initially, and screwed home when the adjustment has been made.

(iv) Leave the standard solution in the left hand cup. Tip back or throw away the solution from the right-hand cup, draining out as much as possible. It is not necessary to dry out the cup completely, as one drop of standard added to several cubic centimetres of unknown will introduce no appreciable error. Fill the right hand cup with the unknown, replace, and rack up or down till the unknown matches the standard.

Some workers take a single reading, but most prefer to take five or six and average the results. Prolonged staring must be avoided lest retinal fatigue occurs. The first reading is commonly slightly different from the rest, and in that case may be discarded. It is found that there are often slight variations in the readings obtained by different individuals. Thus my assistant almost always obtains a slightly higher average reading than I do, though both of us have had ample practice. This personal factor, and the factor of retinal fatigue, have both been raised as objections to the colorimetric method, but actually it is found that the variation expressed as a percentage of the final result is very small, usually less than 2 per cent. In volumetric titrations, two workers may not vary by more than 1 drop, whereas their colorimetric readings may differ by 2 or 3 mm, but to compare the difference between two colorimetric readings with the difference between two titration figures both must be expressed in common terms.

To simplify the calculation the unknown may be set at a fixed level (e.g., 20 mm) in the left hand cup, and the standard may be placed in the right hand cup and moved up or down to match the unknown (cf. calculation of blood sugar (Folin and Wu) in Chapter VII).

(v) Calculation —

Concentration of substance in volume of unknown solution employed	= $x$
Reading (depth in millimetres) of "unknown"	= $u$
Concentration of substance in volume of standard solution employed	= $y$
Reading of "standard"	= $s$

$$x \times u = y \times s, \text{ or } x = \frac{s}{u} \times y,$$

wherefore  $x$  is readily calculated, since  $y$  is known, and  $s$  and  $u$  are measured. If standard and unknown are each made up to the same volume, which is the usual procedure, then  $y$ , and hence  $x$ , can be expressed directly in terms (say) of milligrammes per 100 c.c., but otherwise allowance must be made in the calculation for the relative volumes of standard and of unknown.

The intensity of colour is directly proportional to the concentration of the substance responsible for the colour.

(vi) Notes. Some chemicals attack the varnish on the shoulders of the plungers. Do not, therefore, overfill the cups.

In the Kober colorimeter the base of the cup is fused on, the cups may, therefore, be used for almost any substances. In some types of colorimeter, however, the base of the cup is cemented on, in which case chemicals which attack the cement must not be used.

When badly stained, opal or frosted glass mirrors may be cleaned with fine emery or carborundum paste.

In some instruments the fields are crossed, the optical arrangement being such that the right half of the field corresponds to the solution in the left cup, and the left half to the right cup. Although this sometimes puzzles the beginner, it is immaterial, because the scales move with their corresponding cups.

### Nephelometer

This is an instrument for comparing the ratio of the opacities of two suspensions. A colorimeter may generally be adapted for use as a nephelometer. Different types of cups must be used. A colorimeter cup has dark opaque walls and a clear transparent bottom. A nephelometer cup has clear transparent walls and a dark opaque bottom. In a colorimeter the light comes through the bottom of the cup and up through the solution into the plunger. In a nephelometer the light passes directly through the walls of the cup, and is reflected by the particles of the suspension up through the plunger. The Kober pattern is a combined colorimeter nephelometer.

The main difficulty in nephelometric methods is to ensure that the same state of aggregation of particles is obtained in standard and in unknown. Since the standard suspension is obtained by adding certain reagents to a simple solution of a given substance, whereas the unknown suspension is obtained by adding the same reagents to a complicated solution (e.g., protein free filtrate of blood), this difficulty is readily appreciated.

Nephelometric methods are not often used in chemical pathology and in general are not trustworthy when applied to biological fluids.

## CHAPTER II

### THE ROUTINE CHEMICAL EXAMINATION OF THE URINE (Qualitative)

WHAT is the minimum permissible in the routine examination of the urine? This is the question that faces the bewildered student early in his career in the wards. The busy practitioner and the laboratory worker are often faced with the same problem. It is just as essential to examine the urine as, say, the chest in routine clinical investigations, but how often are urinary examinations omitted? Possibly such omissions would be fewer if a routine procedure could be adopted which was simple, and yet ensured that nothing of importance would be missed. The following scheme is suggested —

- ❖(1) Naked eye appearances
- (2) Reaction to litmus
- (3) Tests for protein ("albumin")
- (4) Tests for reducing substances ("sugar")

Just as there are four stages in chest examinations—inspection, palpation, percussion and auscultation—so one may memorise the four steps of the minimum routine urinary examination as inspection, reaction, protein and reduction.

Under (1) will be noted the colour of the urine, whether it is clear, opalescent or cloudy, whether there is any deposit visible, and, if so, the naked eye appearance of the same. Urines abnormal in colour are dealt with in Chapter XI, and urinary deposits in Chapter IV.

As regards (2), the only point which may cause confusion is the term "amphoteric". It is sometimes stated that on occasion the same urine may turn both red litmus paper blue and blue litmus paper red, and that such urine is "amphoteric" in reaction. This statement is apt to be misleading.

The colour of litmus changes from red in a definitely acid solution ( $pH$  5.4) through varying shades of reddish purple and purple as the solution becomes less acid, to blue when the reaction is definitely alkaline ( $pH$  7.8). When the urine is intermediate in reaction (about  $pH$  6.8) red litmus paper is changed to a reddish purple tint, and blue litmus paper is changed to the same intermediate tint. The student may demonstrate these changes of tint for himself by testing fresh milk with red and blue litmus paper. Fresh cow's milk has a reaction of about  $pH$  6.8, and is also said to be "amphoteric" in reaction.

(3) and (4) Tests for protein are considered in Chapter III, and tests for reducing substances in Chapter VI. It should be an invariable rule to examine microscopically the deposit (centrifuged if possible) whenever proteinuria is found. Similarly, when reducing substances are discovered in the urine, the specimen should automatically be tested for acetone bodies. Our scheme, therefore, becomes —

- (1) Naked eye appearances { Colour  
Deposit  
(2) Reaction to litmus  
(3) Tests for protein ("albumin") —→ (5) Examination of  
centrifuged deposit  
(4) Tests for reducing substances  
("sugar") —→ (6) Test for acetone  
bodies

The examination of the deposit is outlined in Chapter IV, and tests for acetone bodies in Chapter IX.

The above scheme will be found to be a good working minimum in practice. The colour of the urine, points in differential diagnosis, etc., will often call for additional tests which it is not essential to include in the original routine examination. It will be noted, for instance, that so far tests for blood have not been mentioned. The reason is that, with the above scheme, if blood is present in large or moderate quantities, the abnormal colour alone will make obvious the necessity for tests for blood. In any case, even if the amount of blood be very small, protein will be found, and that will be followed by a microscopical examination of the centrifuged deposit, which is the best test for hæmaturia (see Chapter X). Similarly, a yellow skin or yellow sclerotics or the colour of the urine will suggest testing for bile (Chapter XII), and so on.

In laboratory work it is valuable to enlarge this scheme of routine examination by three further tests, viz. those for urobilin (and urobilinogen), indican, and chlorides (qualitative test). Tests for urobilin are given in Chapter XII. The simplest is the spectroscopic examination of the urine after filtration and acidification with a mineral acid. Incidentally, spectroscopic examination (see Chapter X) may reveal the presence of other abnormal constituents, *e.g.*, hæmoglobin, porphyrin, etc. Whilst indican (Chapter XIV) is being tested for, the presence of bromides or iodides in the urine (Chapter XI) may incidentally be noted. Chlorides are discussed in Chapter XV.

Though not essential in the examination of every urine, this third scheme may also be helpful to the student and practitioner —

- (1) Naked eye appearances { Colour  
Deposit  
(2) Reaction to litmus  
(3) Tests for protein ("albumin") —→ (5) Examination of  
centrifuged deposit  
(4) Tests for reducing substances  
("sugar") —→ (6) Test for acetone  
bodies  
(7) Tests for urobilin and  
urobilinogen —→ (8) Spectroscopic  
examination  
(9) Tests for indican  
(10) Qualitative test for chlorides

Lastly, it is often valuable to have a scheme for recording results which shall omit nothing essential. In looking up records of past cases, the failure to record negative findings often leads to doubt as to whether search has been made for a particular product. The following form has been in use in the writer's laboratory for several years, and its routine adoption has, not infrequently, led to the discovery of pathological conditions unsuspected by clinical colleagues —

---

NAME	Age	Lab Ref No
WARD		Date
Phys or Surg		Clin Diagnosis

---

### URINE ROUTINE CHEMICAL

---

Colour	Urobilin and/or ogen												
Specific gravity	Bilirubin												
Reaction	Bile salts												
Total protein	Indican												
"Sugar"	Chlorides (qual <sup>re</sup> test)												
Acetone bodies	Other findings												
Deposit { <table> <tr><td>W B C</td></tr> <tr><td>R B C</td></tr> <tr><td>Ep cells</td></tr> <tr><td>Mucus</td></tr> </table>	W B C	R B C	Ep cells	Mucus	<table> <tr> <td rowspan="5">Casts {</td> <td>Hyaline</td> </tr> <tr><td>Granular</td></tr> <tr><td>Epithelial</td></tr> <tr><td>Blood</td></tr> <tr><td>Waxy</td></tr> <tr> <td>Crystals</td> <td></td> </tr> </table>	Casts {	Hyaline	Granular	Epithelial	Blood	Waxy	Crystals	
W B C													
R B C													
Ep cells													
Mucus													
Casts {	Hyaline												
	Granular												
	Epithelial												
	Blood												
	Waxy												
Crystals													

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### PRACTICAL INSTRUCTIONS FOR ROUTINE TESTS <sup>1</sup>

1 Note *reaction to litmus*, the *specific gravity*, and the *colour* of the urine, and the *naked eye appearance of the deposit* (if any)

#### 2 Protein

**Boiling Test** Fill a test tube three parts full with clear urine (filtered if necessary), incline at an angle and boil the upper layer by means of a small flame. A turbidity indicates either protein or earthy phosphates. Add 1 drop of acetic acid (33 per cent) and boil again. Any remaining turbidity indicates the presence of protein.

*Note.* If excess of acetic acid be added to an acid urine a small amount of protein may be missed, owing to its conversion into metaprotein on boiling in acid solution—hence the reason for adding only 1 drop of acetic acid. If the reaction of the original <sup>(2)</sup> urine be alkaline 1 drop of acetic acid may not be sufficient to render the mixture acid, in which case the protein may not be coagulated. Large amounts of protein may be missed in this way. If, therefore, the reaction of the original urine be alkaline to litmus,

<sup>1</sup> For more detailed discussions of these tests see subsequent chapters

make it slightly acid before performing the boiling test. Or, alternatively, after boiling the original urine, continue the addition of the acetic acid until the reaction is slightly acid and boil again.

**Salicyl-sulphonic Acid Test** Into a  $\frac{1}{2}$  in test tube pour enough clear urine (filtered if necessary) to form a column approximately 1 in high. Add 10 drops of 25 per cent salicyl sulphonic acid. A white precipitate indicates the presence of protein.

**Notes** If there is any difficulty in deciding whether a precipitate has formed, compare the opalescence of the treated urine with a second sample of the original untreated urine placed in another test tube.

If the urine is very alkaline more salicyl sulphonic acid will be required.

Very occasionally false positive reactions may be due to uric acid or to uroselectan (see p 232).

**Nitric Acid Ring Test** Place about 3 c.c. ( $\frac{1}{2}$  in column) of concentrated nitric acid in a test tube, and superimpose about 3 c.c. of urine. A white ring generally indicates protein (albumin, globulin, mucus or proteose), but occasionally may be due to urea nitrate, uric acid, resinous bodies, uroselectan or even bile acids. The nitric acid may oxidise pigments or chromogens so that a dark ring is formed at the junction of the two fluids, and this may obscure a slight precipitate of protein.

### 3 "Sugar" (Reducing Substance)

**Benedict's Test** Transfer to a  $\frac{1}{2}$  in test tube enough Benedict's qualitative solution to make a column approximately 1 in high. To this add 8 drops of the urine. Boil vigorously for two minutes and (if doubtful) allow to stand till the precipitate settles. If "sugar" is present a red or yellow precipitate of cuprous oxide will be observed.

**Note** It is essential to add a small volume of urine and to boil thoroughly. If too much urine be added the results are apt to be ambiguous, owing to a precipitate of earthy phosphates. Even with the 8 drops recommended a slight white or greyish precipitate of earthy phosphates may sometimes appear and simulate a feeble reduction.

**Fehling's Test** Take two test tubes and in the one (a) place approximately 5 c.c. (1 in column) of urine and in the other (b) an equal volume of Fehling's solution. Heat the two tubes over the same flame and, when boiling, add the contents of (a) to (b). Allow the mixture to stand without further heating. If an abnormal amount of "sugar" is present, a red or yellow precipitate will appear.

**Note** Prolonged boiling of the urine with Fehling's solution is very apt to lead to the formation of a greenish precipitate owing to the action of the strong alkali on normal urinary constituents.

**Nylander's Test** (reduction of bismuth salt) To about 5 c.c. (1 in column) of urine in a test tube add one fifth its volume of

Nylander's reagent, and boil thoroughly for about two minutes. A black precipitate of bismuth will appear rapidly if much "sugar" is present. Otherwise place the tube aside for a few minutes and note whether a black precipitate settles to the bottom. White or greyish white or brownish precipitates are of no significance.

#### 4 Acetone Bodies

**Rothera's Test for Acetone and Aceto-acetic Acid (Modified)**  
Place in a test tube the mixed crystals of ammonium sulphate and sodium nitroprusside (powdered in proportion of 100 parts of sulphate to 1 part of nitroprusside) to a height of  $\frac{1}{2}$  in (about 2 gm), and add urine to fill the tube half way (about 10 c c). Then add 2 or 3 c.c. ( $\frac{1}{2}$  in column) of concentrated ammonia place the thumb over the mouth of the test tube, shake well, and allow to stand. A characteristic purple (permanganate) colour indicates the presence of acetone, or aceto acetic acid, or both.

The test is much more sensitive for aceto acetic acid than for acetone. The intensity of the colour and the rate at which it develops vary with the concentration.

**Gerhardt's Test for Aceto acetic Acid**—To approximately 5 c c (1 in column) of the urine in a test tube—add 10 per cent ferric chloride, drop by drop till no further precipitate of ferric phosphate is formed. Filter. To the filtrate add some more ferric chloride. A Bordeaux red colour indicates aceto acetic acid. Be careful to avoid excess of ferric chloride lest the red colour be masked.

**Note** A similar colour is given by a large number of substances, such as salicylic acid, and the bodies excreted after administration of aspirin, antipyrin, thallin, etc. The majority of these substances are not destroyed by boiling, whereas aceto acetic acid is converted into acetone and CO<sub>2</sub> if the urine be boiled thoroughly, e.g., in a boiling tube or conical flask.

#### 5 Blood

**Examination of Centrifuged Deposit (Hæmaturia) and Use of Spectroscope (Hæmoglobinuria)** By far the most satisfactory test for blood is the macroscopical and microscopical examination of the centrifuged deposit. This deposit may be red to the naked eye, although the original urine be not abnormal in colour. The presence of red blood corpuscles is obvious on microscopical examination, even when the blood is so highly diluted that the most delicate chemical test fails to reveal its presence. If on spectroscopic examination of the urine cleared by centrifuging the two characteristic absorption bands of oxyhæmoglobin (see Fig 47, facing p 212) are seen, hæmoglobin is present in solution in the urine.

**Guaiacum Reaction** Boil well about 5 c c (1 in column) of urine in a test tube, cool and add 2 drops of fresh tincture of guaiacum. Shake, and float a layer of ozonic ether on the surface. When blood is present a blue ring is formed (a blue ring is also given by pus and by the urine of patients taking iodides). The

test may not be positive when blood is present in small quantities although red blood corpuscles can easily be seen with the aid of a microscope

**Reduced Phenolphthalein Test** To about 3 c.c. ( $\frac{1}{2}$  in column) of reduced phenolphthalein (Kastle Meyer reagent—see Appendix) add 10 drops of 10 volumes hydrogen peroxide. Then add an equal volume ( $\frac{1}{2}$  in column) of urine, and shake. Do not warm. If blood be present the mixture assumes a pink colour. It is essential that the test tubes be absolutely clean. The reagent becomes less sensitive on keeping, it must be filtered if not absolutely clear.

**Pyramidone Ring Test** Acidify 2 to 3 c.c. of urine in a test tube with a few drops of 33 per cent acetic acid. Carefully superimpose about an equal volume of 5 per cent pyramidone (amidopyrin) in absolute alcohol. Add 5 to 6 drops of 10 volumes hydrogen peroxide, allowing each drop to fall well into the solution. A lilac or mauve ring indicates blood. (If the urine contains iodides a "false positive" results owing to the liberation of iodine.)

**Hyperol-Benzidine Test** (J. Ingham, *Biochem. J.*, 1932, 26, 1124) On a white tile mix a knife point of solid benzidine, 2 drops of glacial acetic acid, 1 drop of urine and a knife point of hyperol (solid compound of  $H_2O_2$  and urea, 35 per cent  $H_2O_2$ ). A blue colour results if blood be present, even if only in traces. Pus, enzymes and iodides give false positive reactions.

## 6 Bile

**Bile Salts Hay's Test** Sprinkle the surface of some urine in a test tube with finely powdered flowers of sulphur. The particles fall to the bottom of the tube if bile salts are present. Treat a normal urine similarly for comparison.

**Bilirubin Iodine Test** Perform a ring test, floating tincture of iodine (see Appendix) diluted with an equal quantity of distilled water on the surface of the urine. A green ring indicates bilirubin.

**Gmelin's Test** Place about 3 c.c. ( $\frac{1}{2}$  in column) of concentrated nitric acid in a test tube, and superimpose about 3 c.c. of urine. A green or blue ring indicates bilirubin.

Alternatively the test may be applied by adding a drop of concentrated nitric acid to a piece of filter paper previously soaked with the urine. Or a drop of each fluid may be placed close to one another on a white porcelain tile and the drops run together by tilting the tile. The play of colours is noted green or blue being characteristic of bilirubin.

**Fouchet's Test applied to urine** To a test tube half filled with acid<sup>1</sup> urine (about 10 c.c.) add half the volume (about 5 c.c.) of 10 per cent barium chloride<sup>2</sup>. Mix and filter. Unfold the paper, and spread it on another dry filter paper. Allow 1 drop of Fouchet's reagent (see Appendix) to fall on to the precipitate. A green

<sup>1</sup> If alkaline acidify with acetic acid.

<sup>2</sup> If there should be little or no precipitate, add 1 or 2 drops of saturated ammonium sulphate solution.



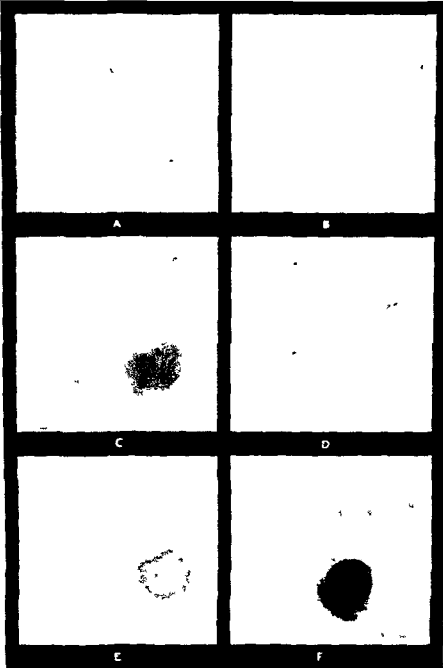


FIG 4 FOUCHET'S TEST APPLIED TO URINE.

A Test negative Normal urine The barium precipitate was practically white, and the drop of reagent caused no colour change

B Test negative Urine contained urobilin, but no bilirubin The barium precipitate was yellow brown, but the treated spot did not develop any green or blue

C Test positive Urine contained urobilin and bilirubin The barium precipitate was very pigmented, but the treated spot did not develop a correspondingly intense blue

D Test weakly positive Urine contained a little bilirubin The barium precipitate was pale yellow, and the treated spot was pale blue

E Test more strongly positive than D Urine contained a little more bilirubin

F Test strongly positive The barium precipitate was deep yellow and the treated spot deep green

(biliverdin) or blue (cholecyanin) colour shows that bilirubin is present (see colour plate Fig 4)

This test is more sensitive than the first two, and is quite as delicate as the next

**Hunter's Diazo Test** (*Canadian Med Assoc J*, 1930, 23, 823)  
In a centrifuge tube place 10 c.c. of urine, if it is not acid, it must be made slightly so with acetic acid. Add 4 c.c. of 10 per cent barium chloride, mix, and mark the upper level of the fluid with a grease pencil. Centrifuge and decant completely. Add distilled water to the mark, mix thoroughly, centrifuge and decant completely again. Stir the precipitate with 0.5 c.c. of Van den Bergh's diazo reagent (see p 262), by means of a capillary pipette of which the end has been sealed in the flame. Add 2 c.c. of alcohol (absolute or 96 per cent) and 0.3 c.c. of 6 per cent  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ . Mix and centrifuge. If bilirubin is present the supernatant fluid will be red, due to the formation of azobilirubin.

Since this test depends on a different principle from the other three, it is valuable, and particularly in cases of doubt. The quantities given must be closely adhered to, otherwise the sensitivity of the test may be considerably reduced, this limits its use in every day work.

## 7 Urobilin and Urobilinogen

**1) Spectroscopic Examination** Acidify some of the urine with hydrochloric acid and examine with the spectroscope. If urobilin is present, a characteristic absorption band will be seen at the junction of the green and blue (see Fig 48 facing p 220). Normal urine shows no absorption bands.

**2) Schlesinger's Test** To about 10 c.c. of urine (2 in column) in a test tube add 6 drops of tincture of iodine,<sup>1</sup> which changes urobilinogen into urobilin. In another tube place about 10 c.c. of absolute alcohol and about 1 gm. of powdered zinc acetate, roughly measured with a marked small tube (Fig 5). Pour the contents of one tube into the other, pour the mixture back into the empty tube and repeat the process till the solid zinc acetate has mostly gone into solution. Filter, and examine the filtrate first directly by transmitted light, and secondly by reflected light with your back to the window, or view it from above, looking down the tube. A green fluorescence is due to a compound of zinc with urobilin. Normal urines show no appreciable fluorescence. Always confirm the presence of urobilin by examining the filtrate with the

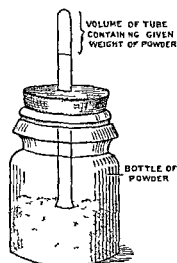


FIG 5 Marked tube fixed in stopper for approximate measurement of powders

<sup>1</sup> The tincture diluted with water as used above in testing for bilirubin, is convenient and satisfactory

spectroscope for the characteristic absorption band at the junction of the green and blue. If the urine contains much bilirubin, as well as the urobilin, the fluorescence may be masked, but the absorption band of urobilin is clearly visible on spectroscopic examination.

**Bogomolow's Test** Treat half a test tube full of urine with 10 drops of 20 per cent copper sulphate. Add 3 or 4 c.c. ( $\frac{1}{2}$  in column) of chloroform, place the thumb on the top of the tube and invert ten times without shaking. Allow the chloroform to separate by standing, or, if the emulsion will not break down readily, transfer the chloroform layer with the aid of a capillary pipette to another test tube or separate the layers by centrifuging. Urobilin is present if the chloroform be coloured pink or yellow, but its presence must be confirmed by spectroscopic examination. Bilirubin may colour the chloroform greenish yellow, but gives no absorption band, though it may cause a general absorption of the blue end of the spectrum.

### 8 Indican

**Obermayer's Test** To about 5 c.c. (1 in column) of urine in a test tube add an equal volume of Obermayer's reagent and about 3 c.c. ( $\frac{1}{2}$  in column) of chloroform. Mix by inverting repeatedly, the mouth of the tube being closed with the thumb. If indican is present the chloroform will become blue, due to the extraction of indigo blue. The depth of blue gives a rough measure of the quantity of indican present.

Obermayer's reagent is a 0.2 per cent solution of ferric chloride in concentrated hydrochloric acid. The acid liberates indoxyl from potassium indoxyl sulphate (indican), the indoxyl is oxidised and condensed to indigo blue by the weak oxidising agent ferric chloride.

**Jaffé's Test** Treat a 1 in column of urine with a rather larger volume of concentrated hydrochloric acid and add 3 or 4 c.c. of chloroform ( $\frac{1}{2}$  in column). Mix thoroughly for fully a minute. Allow the chloroform to settle and examine its colour. If it be blue indican is present. Then add a single drop of 1 per cent potassium chlorate and mix thoroughly as before, the chloroform layer may now be colourless, or of a deeper blue depending on the amount of indican. Add a second drop of the chlorate and mix well again. A rough idea of the concentration of indican may thus be obtained by noting the number of drops of potassium chlorate required to oxidise it through the stage of indigo blue to a colourless compound. If no blue be found at any stage indican is absent.

With a mere trace of indican indigo blue is formed only in the first stage before the chlorate is added owing to oxidation by a trace of hypochlorous acid in the hydrochloric acid.

**Note** It is essential to add at least an equal volume of concentrated hydrochloric acid to liberate free indoxyl. This is oxidised and condensed to indigo blue which is soluble in chloroform. If the urine contains iodides the chloroform will be coloured reddish violet by the liberated iodine. Bromides similarly are oxidised.

to bromine—the chloroform turns yellow brown With excess of potassium chlorate, chlorine is formed in quantities sufficient to tinge the chloroform layer yellow Addition of a few drops of 10 per cent sodium thiosulphate and shaking will decolorise iodine but will not alter indigo blue

### 9 Chlorides

To a  $\frac{1}{2}$  in column of urine add 10 drops of concentrated nitric acid and a  $\frac{1}{2}$  in column of 3 per cent silver nitrate Normally, an abundant curdy precipitate of silver chloride appears at once If the chlorides are diminished the solution merely appears milky or opalescent

*Note* If nitric acid is not added, urates might be precipitated, especially if the urine be ammoniacal If protein is present in the urine it will be precipitated by a small quantity of the nitric acid, but will be redissolved on addition of an excess

### 10 Microscopical Examination of Deposit

Whenever proteimuria has been found, the deposit obtained by centrifuging the urine should invariably be examined under the microscope Mount a drop of the deposit under a cover slip, and look particularly for the following —

Cells (w b c , r b c , epithelial c )

Casts (hyaline, granular, epithelial, blood, waxy)

Crystals (calcium oxalate earthy phosphates, uric acid and urates cystine, etc )

Miscellaneous (parasites, ova, mucus foreign bodies, etc )

## CHAPTER III

### PROTEINS IN THE URINE: "ALBUMINURIA"

**BOOKS.** The student will find a full account of tests for protein in *Clinical Methods* by Hutchison and Hunter, and in the manuals of physiological chemistry, etc., e.g., Cole's *Practical Physiological Chemistry*, Emerson's *Clinical Diagnosis*, Hawk and Bergeim's *Practical Physiological Chemistry*, etc. The following is an attempt to simplify the usual accounts and to bring out the essential points of practical importance.

PROTEINS are precipitated by the salts of heavy metals, by the so called "alkaloidal reagents," and by concentrated alcohol. They give certain colour reactions. The globulins and albumins are coagulated by heat.

It is on the basis of these properties that clinical tests for proteins in the urine have been devised. Thus, in Roberts' test, magnesium sulphate, and in Spiegler's test, mercuric chloride, are used as the protein precipitants. Phosphotungstic acid, phosphomolybdic acid, metaphosphoric acid, and picric acid are examples of the so called alkaloidal reagents. Numerous tests for protein in the urine are given in text books of physiological chemistry, but in clinical medicine only two or three of these are commonly used. The student is advised to employ these commoner tests, and to learn by personal experience the difficulties of technique, and the interpretation of the results obtained.

### QUALITATIVE TESTS FOR PROTEINS IN URINE

(For practical instructions, see Chapter II.)

**Boiling and Acetic Acid Test.** The proteins are coagulated by boiling. Carbon dioxide is driven off by the heat, and in some cases the resulting shift in the reaction of the urine to the alkaline side may be sufficient to bring about a precipitation of earthy phosphates. Therefore acetic acid is always added to redissolve any precipitated phosphates. ①

Strictly speaking the urine should always be made just acid to litmus before boiling. This, however, is rarely done in clinical work, and as a result erroneous conclusions are not uncommonly drawn. If the reaction to litmus is taken as a routine (see Chapter II) the possibility of such errors is less likely to be forgotten. If the urine is very alkaline (and samples provided for testing fairly commonly are alkaline owing to the ammoniacal decomposition which takes place on standing), the boiling may not bring down the protein. On addition of 1 or 2 drops of acetic acid the protein

may then be coagulated. This observation has often come as a surprise to the student. What happens is this. Owing to the action of the alkali in the untreated urine, accelerated by the heat, the protein is changed to metaprotein, which is soluble in alkali, and therefore is not coagulated. (Only a precipitate of metaprotein is coagulated by heating.) When sufficient acetic acid is added to neutralise the alkaline urine the metaprotein is precipitated (metaprotein is insoluble in solutions of neutral salts) and is immediately coagulated by the heat. It sometimes happens that the urine is so alkaline that the addition of even 5 to 10 drops of 33 per cent acetic acid after boiling may not cause coagulation. Specimens may be reported erroneously as protein free for this very reason.

Similarly, if the original urine is very acid the protein is changed into metaprotein, which is soluble in acid, and is, therefore, not coagulated. In clinical work, however, it is rare for the natural reaction to be so acid as to cause the presence of protein to be missed in this way, though of course it may be brought about by careless over acidification before heating.

Cole recommends the addition of brom cresol purple to the original urine, correcting the reaction, if necessary, before heating, and again after heating by addition of acetic acid till the colour becomes yellow. (Brom cresol purple is purple in alkaline, yellow in acid solution.)

The coagulum or precipitate obtained on boiling and the subsequent addition of acetic acid, may be albumin, pseudo-globulin, eu or lipid globulin ("nucleoprotein"), or mucus. Very rarely resinous bodies may be thus precipitated. A discussion of the various proteins is given below.

① The Salicyl-sulphonic Acid Test. Albumin, globulins, mucus, proteoses and Bence-Jones' protein are all precipitated by salicyl-sulphonic (sulphosalicylic) acid. This is a very simple and excellent test for proteins. The only difficulty occurs in testing urines which are slightly opalescent. This may be overcome by comparing the treated urine in one test tube with the untreated in another held by the side of the first.

Very occasionally the reagent precipitates uric acid, this is easily distinguished because it redissolves on heating to about 60° C. Urine containing uroselectan (sodium salt of 5 iodo 2 pyridone N acetic acid) gives a white crystalline precipitate (see p 232).

Exton employs 5 per cent. salicyl sulphonic acid in 20 per cent crystalline sodium sulphate instead of 25 per cent salicyl sulphonic acid. He mixes equal parts of his reagent and of the urine, and warms.

② Heller's Nitric Acid Test. This is performed as a ring test and is very delicate, revealing the presence of 0.007 per cent of protein, or even less. The protein is changed into metaprotein by the nitric acid. Metaproteins are insoluble in concentrated mineral acids. In the writer's experience this test not uncommonly misleads

the student for one of the reasons given below. The salicyl sulphonic acid test is recommended in preference.

The white precipitate formed on the addition of nitric acid may be albumin, globulin, mucus, proteoses, urea nitrate, uric acid, resinous bodies, uroselectan acid (see p. 232), or even bile acids (in concentrated urines). Urinary pigments or chromogens are oxidised by nitric acid to darker compounds, and when proteinuria is slight the white ring may be obscured by the dark pigments. The precipitation of urea nitrate, of uric acid, and of bile acids may be prevented by a preliminary dilution of the urine, e.g., 1 in 3 with physiological saline. Precipitates of urea nitrate and of uric acid may dissolve on warming, but on so doing the ring formation is destroyed. Similarly, proteoses disappear on heating to reappear on cooling. On adding urine containing uroselectan to the nitric acid, a white precipitate forms at once, followed by a fairly vigorous reaction and solution of the precipitate. Resinous bodies are soluble in alcohol, but care should be taken lest the vigorous reaction between alcohol and nitric acid leads to a minor explosion! This may be avoided by transferring to another test tube some of the turbid urine from the zone immediately above the nitric acid with a capillary pipette. Alcohol or ether may then be added, when turbidity due to resins will disappear.

**Note on the Necessity of Filtering Cloudy Urines** Theoretically, of course, the urine should always be filtered if not absolutely clear before testing for protein. In practice, however, such filtration is often omitted. A slight turbidity causes no difficulty unless proteinuria be very slight, using either the boiling and acetic acid test or the salicyl sulphonic acid test with comparison tube, as suggested above. Moreover in pathological work filtration is frequently useless to clear completely a turbid urine, such turbidity being due to bacteria. It is often recommended that organisms be removed by shaking the urine with powdered barium carbonate and filtering, or by making the urine alkaline with caustic soda till a precipitate of phosphates is formed and then filtering. In practice, though the turbidity is greatly reduced, it is frequently not entirely removed by the above procedures, even though fine filters are used. The writer does not recommend these methods except in very exceptional cases of slight proteinuria, owing to their being so time consuming. A marked turbidity must, of course, be removed by filtration.

## THE QUANTITATIVE DETERMINATION OF PROTEIN IN THE URINE

(For technical details, see end of chapter.)

### (1) Estimation of Total Protein

The method used most commonly in clinical work is that of Esbach. The proteins in a known volume of urine are precipitated by picric acid. The precipitate is allowed to settle by gravity, and

the volume it occupies at the end of twenty four hours is read off. The Esbach tubes are calibrated by noting the volume occupied by the precipitate from protein solutions of known concentration under similar conditions. The method is a convenient one for clinical purposes, but is far from accurate. As a rough method for demonstrating gross changes in proteinuria it is serviceable, but the student should attach no significance to small changes, e.g., an increase or decrease of 1 or 2 parts per 1,000.

Aufrecht's method is the same in principle but is more convenient for laboratory purposes. The protein precipitate is thrown down in a graduated centrifuge tube, thus permitting a reading in a few minutes instead of twenty four hours. It is essential, however, to centrifuge for the prescribed time at the prescribed rate, otherwise hopelessly inaccurate results will be obtained.

Other methods involve gravimetric (e.g., Scherer's method), or nephelometric (e.g., method of Folin and Denis) determinations. Gravimetric methods are the most accurate but are tedious and are not applicable to urines containing small amounts of protein. Nephelometric methods are not really satisfactory owing to the uncertainty of obtaining similar states of aggregation of protein particles in unknown and standard. For clinical work a series of protein standards may be prepared as in Mestrezat's method for estimation of protein in the cerebrospinal fluid (see Chapter XX) and the unknown compared with these. The colour of the urine may easily be compensated for by backing the standard with some of the untreated urine. The use of a comparator facilitates the procedure.

Kerridge utilises the same principle with a slight modification. Peters and Van Slyke conclude that a micro-Kjeldahl determination of the nitrogen of the protein after separation and washing free from non-protein nitrogenous substances, is only slightly less accurate than gravimetric estimation but this is too time consuming for routine work, so the reader is referred to the authorities quoted. Hiller, McIntosh and Van Slyke have published a useful colorimetric method depending on the buret reaction, a modification of which is described at the end of this chapter.

## (2) The Differential Estimation of Proteins Albumin-Globulin Ratio

The proteins are separated into different fractions, and the protein in each fraction is estimated. For separating mucus an excess of acetic acid may be used (in the cold). Globulins may be thrown out by saturation with magnesium sulphate, by half saturation with ammonium sulphate, or by 22 per cent sodium sulphate (Howe).

In 1890 Noel Paton separated the proteins in urine with the aid of magnesium sulphate. He first estimated the total protein (albumin plus globulin (plus mucus)) in the original urine, and then threw out the globulin (plus mucus) with magnesium sulphate and estimated the albumin in the filtrate, he used Esbach's method



for estimating the protein in the two fractions. Recently the differential estimation of proteins in urine has again been studied, but usually no effort has been made to separate mucus from the globulins. Thus Hiller, McIntosh and Van Slyke perform the separation with sodium sulphate and estimate the protein in the fractions colorimetrically (biuret method—see end of chapter). These authors have published an interesting series of results in nephritis. In nephrosis they find the quotient  $\frac{A}{G}$  high, generally above 10, in acute nephritis  $\frac{A}{G}$  was between 5 and 10, in chronic nephritis mostly below 5, and in renal amyloid disease it was the lowest of all (0.5 to 5.0) owing to the high output of globulin. These quotients or "ratios" are the averages of estimations made over a period of several days. The variation of the ratio from day to day is fairly large, and a single test on one day only is of little or no value. For this reason  $\frac{A}{G}$  determinations are of very limited use clinically.

### THE TERM "ALBUMINURIA"

In theory the term "albuminuria" as used in clinical medicine indicates the presence of plasma albumin, plasma globulin, or both, in the urine. As a rule no attempt is made to differentiate between albumin and globulin the significance of the two in general being the same. In practice however, "albuminuria" generally means that tests for proteins in the urine have been positive. "Albuminuria," therefore, really means that one or more of the following proteins is present, viz., albumin, globulin, hæmoglobin, or mucus and "Proteinuria" would therefore be a better term. Proteoses will not be included owing to the fact that they disappear on heating to reappear on cooling.

The student cannot be reminded too often that there are many causes of 'albuminuria' in addition to nephritis.

### THE URINARY PROTEINS

Normal urine contains no proteins apart from a minute trace of mucus (see below), which is not enough to give a positive reaction with the routine qualitative tests.

The different proteins which may appear in the urine under pathological conditions include —

Plasma albumin

Plasma globulin (pseudo globulin, and eu globulin or liquid-globulin ("nucleoprotein"))

Hæmoglobin and methæmoglobin (see Chapter X)

Mucus

Fibrin

Proteoses, and Bence Jones' protein

For details of the properties of each of these proteins the reader is referred to standard works on physiological chemistry. Here only those characters utilised in their detection and separation in clinical medicine will be noted.

Recent work (Svedberg and others) on the determination of the molecular weights is of interest however, because Bayliss, Kerridge and Russell have suggested that the healthy kidney is permeable only to proteins with a molecular weight below about 68,000, and that excretion is governed by the size of the molecules, and is uninfluenced by whether the protein is "foreign" to the body or not.

Proteins which are excreted	Molecular weight	Proteins which are not excreted	Molecular weight
Gelatin	35,000	Hæmoglobin	68,000
Egg albumin	34,500	Serum albumin	67,500*
Bence Jones'	35,500	Serum globulin	103,800
Hæmoglobin	68,000	Casein	188,000
		Edestin	208,000
		Hæmocyanin (Helix)	5,000,000

\* Over 70,000 by osmotic pressure methods

Hæmoglobin is near the border line, and is peculiar in that it is only excreted when its concentration in the plasma exceeds a certain value.

• Plasma albumin is soluble in distilled water and is precipitated by complete saturation with ammonium sulphate. Pseudo globulin is soluble, but eu globulin is insoluble in distilled water. Both globulins are precipitated from solution by half saturation with ammonium sulphate, or by full saturation with magnesium sulphate. Hæmoglobin is decomposed and coagulated by heat. It is usually confined within the red blood corpuscles in the urine ("hæmaturia"). Less commonly it is free in solution ("hæmoglobinuria"). In hæmaturia it is only necessary to separate the red blood corpuscles by centrifuging in order to remove the hæmoglobin (most filter papers allow some red cells to go through). It is probable that true nucleoproteins are only very rarely passed in appreciable quantities in the urine. The term "nucleo proteinuria" in clinical medicine generally signifies that proteins are present which are precipitated by acetic acid in the cold. In most cases, without doubt, the chief protein precipitated by acetic acid in the cold is mucus (mucin). Mucus often occurs in normal urine in minute traces and often separates as a cloud on standing. In all catarrhal conditions of the urinary passages, and particularly of the bladder or prostate, mucus may appear in the urine in varying amounts (usually small). It must not be forgotten that in females mucus not infrequently gains access to the urine from the vagina. In males proteinuria may sometimes be due partially or entirely to admixture with semen. The detection of spermatozoa in the microscopical examination of the deposit will lead to the rejection

of the particular specimen for test purposes<sup>1</sup> Mucus is insoluble in an excess of acetic acid, whereas lipoid globulin, which is also precipitated by acetic acid in the cold, is soluble in an excess of the reagent It is generally held in medicine that proteins precipitated by acetic acid in the cold are of little or no import That is why it is necessary to be able to recognise and separate them Such proteins are commonly found in the intermittent proteinurias of childhood and adolescence, and occasionally in the later stages of a nephritis which is clearing up Lipoid-globulin is most likely to be met with in chronic parenchymatous nephritis ("nephrosis") at a stage when there is marked hypercholesterolaemia. Fibrinuria occurs when there is much blood in the urine so that clotting takes place It is a rare symptom in papilloma of the bladder, sometimes follows the administration of cantharides, and may be found in chyluria (p 284) The fibrin is easily removed by centrifuging or by filtration Proteoses and Bence-Jones' protein are precipitated below 60° C, but disappear on boiling They may, therefore, be separated from other proteins by coagulation at 60° C and filtration In Bence Jones' proteinuria the urine becomes turbid at 40° to 50° C, the precipitate flocculating at 60° C or below "Albumin" starts to come down at about 70° C and flocculates at 80° to 100° C, depending on its concentration Proteoses<sup>2</sup> may appear in the urine when absorption of partially digested pus is proceeding somewhere in the body, and during involution of the puerperal uterus They usually are found in traces only, are often transient, and generally are of little or no clinical significance Bence-Jones' proteinuria on the other hand, though uncommon, is important, because it is practically diagnostic of multiple myelomatosis of the bone marrow, though it has been described in other diseases of the marrow (For technique of recognition, see end of chapter) Indeed, its discovery in the urine may be essential to establish the diagnosis because not uncommonly radiograms give no hint of the marrow changes At one time Bence Jones' protein was regarded as an albumose (proteose), but is now considered to be more closely allied to the globulins The protein contains all the amino acids found in other typical proteins, but is relatively rich in those of the aromatic series (Hopkins and Savory) It is probable that there is more than one type judging from immunological (Bayne Jones and Wilson) and physico chemical properties (Hewitt), but the term Bence Jones' protein can fairly be retained for a quite distinct group of closely allied proteins A little amorphous protein may be deposited in some urines kept for a day or two, and on a few occasions the protein has been crystalline in the form of spherical clusters of needles It is generally believed that it originates from

<sup>1</sup> The proteins of semen are reported to include albumin a nucleoprotein mucin and an albumose like substance (Marshall) The last is probably a protamine (see Rosenheim)

<sup>2</sup> There is no evidence that the so called proteose separated by acid and ether from the urine in asthma and other allergic conditions is a proteose at all and it is unfortunate that it was so labelled

the bone marrow, though it has not always been recovered from that site post mortem. The amount excreted is often relatively large (3 to 70 gm per diem), though probably this depends to some extent on the stage at which the subject first comes under observation. The daily excretion may be very steady in a given individual for a period of months, and generally is not significantly altered by variations in the dietary protein, not even by short periods of starvation. For further particulars the reader is referred to Rosenbloom's review and to Peters and Van Slyke's book.

**Contaminating Proteins.** It is important to obtain specimens of urine with the aid of a catheter in any case of obscure proteinuria, to exclude the deliberate addition of protein to the urine, or contamination by semen or by discharges from the vagina or rectum. Such contamination may often be suspected from the microscopical examination of the centrifuged deposit (see Chapter IV). In the case of males the use of the catheter may often be avoided by making the patient pass urine in the presence of the doctor.

**So-called Peptonuria.** Every now and then when urine is added to Fehling's or Benedict's reagents in the test for "sugar," it is observed that the mixture in the cold has a deeper blue, or purplish colour, than the original copper reagent. This is often due to the presence of proteins in the urine giving a biuret reaction. But a similar colour change was observed in the absence of proteinuria over fifty years ago (e.g., Ralfe, 1883), and was ascribed to the presence of peptones, when the urine is superimposed carefully on the reagent, a reddish purple or rose coloured ring is observed. Godfried has recently studied this phenomenon, and has shown that it is due neither to peptones nor to urobilin (another suggestion), but to a pigment of the urochrome group, or something associated with urochrome. The point is of interest because the practitioner must come across it sooner or later in his routine urinary examinations, but so far as the writer knows it has no clinical significance.

## CHEMICAL TESTS IN INTERMITTENT PROTEINURIA

Intermittent proteinuria is commonly met with in life insurance work, and in the routine examination of urine, and particularly in adolescents. Proteinuria following scarlatina is not uncommonly intermittent (Russell). It may or may not be strictly postural in type, i.e., it may never appear whilst the individual is in the horizontal position, but only whilst the erect attitude is adopted. In this connection it is important to make sure that the patient empties the bladder one or two hours after retiring, otherwise the early morning urine may contain protein which was excreted and collected in the bladder before going to bed. In order to show that the proteinuria is postural in type, the urine should be collected first thing just before rising again some two or three hours after getting up, and if desired a third or fourth specimen may be obtained later in the day. In typically postural cases the first specimen

should be free from protein, whilst the second will always contain "albumin." The third and fourth samples may or may not contain protein. In some cases the protein disappears after the patient has been up for several hours. Later in the day it may reappear. On account of this observation, objection has been made to the term "postural," and the word "cyclic" has been substituted. Other names that have been suggested are "orthostatic," "lordotic," "functional," "albuminuria of adolescence," etc. In the absence of any clinical signs of organic disease, the demonstration that the proteinuria is postural is of itself suggestive that the proteinuria is of no significance.

But of greater importance is the examination of the centrifuged deposit (see Chapter IV). Normally there should be at the most only some five to ten leucocytes on the whole slide, and an occasional epithelial cell. Squamous epithelial cells are of no significance, being derived from the skin or vaginal mucous membrane. Red blood corpuscles are abnormal, but their source of origin should be decided, e.g., contamination by menstrual flow, hæmorrhage from piles, etc. The single finding of an isolated hyaline cast may be ignored, but as a general rule it is wise to regard the presence of casts as pathological. If, therefore, cells or casts are found persistently in a case of intermittent proteinuria, it is unwise to regard the "albuminuria" as of no significance, though prolonged observation and repeated examination may occasionally justify such a view. On the other hand, if there be no casts or cells (several examinations), it is generally safe to disregard the proteinuria. A not uncommon cause of intermittent proteinuria in men is contamination of the urine by semen.

Many of those individuals with intermittent proteinuria whose urinary deposits contain a few leucocytes and casts show no clinical signs of disease and live to a ripe old age. MacLean found that of the urines of 50,000 healthy soldiers, 0.84 per cent contained definite epithelial casts and 1.03 per cent contained the hyaline variety only. This does not imply, however, that it is safe to disregard the presence of epithelial casts in any one individual case. It is probably wiser to regard such a case as abnormal till repeated examination and prolonged observation have proved the contrary. It is reasonable to adopt the attitude that these patients have very mild organic lesions, and that most of them will recover spontaneously without any treatment. One might place the condition on a par with the common cold. It is very unlikely, though always possible, that the condition of the particular individual under examination will progress and become serious. In this sense the insurance companies are justified in deferring a case until the urine becomes free from cells and casts.

It is often of interest and occasionally of value, to determine the nature of the protein or proteins excreted in intermittent "albuminuria." Sometimes all the protein is precipitated by acetic acid in the cold, but more commonly there is a mixture of proteins including true albumin. Because of these varying

proportions, from a purely practical point of view, the differential estimation of the proteins does not help the clinician. The ratio of albumin to globulin is inconstant and of little or no value (see also p 26).

Renal function tests are very occasionally of value in excluding gross organic lesions, but in the vast bulk of cases of intermittent proteinuria such tests yield "normal" results (Harrison). That does not mean, of course, that the kidneys are necessarily healthy (see Chapter V).

## THE CLINICAL CONDITIONS IN WHICH PROTEINURIA OCCURS

Proteinuria may be one of many clinical signs or may be the only evidence of any abnormality in a subject otherwise normal. It is, of course, simply a matter of words, or of personal choice, whether it be stated that normal urine contains no protein other than a trace of mucus, or whether it be stated that the urine of some healthy individuals contains protein which is of no clinical importance. Some cases of glycosuria provide an exact parallel, thus, glycosuria due to true renal glycosuria is of no moment, but until it has been shown conclusively that the condition is renal glycosuria, the urinary finding may be of serious import. Similarly the medical officer must remember that every case of proteinuria needs investigation before he can be satisfied that it is immaterial. The subject has been discussed under "Chemical Tests in Intermittent Proteinuria."

When there are clinical signs or symptoms in addition to proteinuria, the significance of the proteinuria must be determined in the light of such findings, and upon the conclusions drawn from the whole of the evidence will be inferred what is the probable cause of the proteinuria, and accordingly its course may be predicted.

The compilation of a huge list of all possible diseases and conditions in which proteinuria may occur would clearly be of little or no value, but it is necessary to have some sort of order in thinking of the possible causes and clinical conditions when the qualitative tests for protein in the urine have been found to be positive. The writer's practice is to think in anatomical sequence from the exterior inwards, from possible contamination to lesions of the genito-urinary tract in ascending order, and, finally, to the blood and other sources from which the blood may carry protein to the kidneys. It is obvious that the responsible lesions may be situated in more than one anatomical site, as when both urethra and prostate are involved in gonorrhoea, or when both bladder and kidneys are diseased in tuberculosis, and so on but the anatomical sequence is convenient. The subject will be dealt with in that order now, a few practical points being inserted here and there under the different headings.

Contamination as the cause of proteinuria has already been considered.

Diseases of the External Genitalia or of the Urethra. The

venereal diseases, non specific urethritis, trauma of the urethra, externally or by calculus or gravel, and so on the heading is sufficient

**Diseases of the Bladder or Prostate** Cystitis, tuberculosis, vesical calculi and growths, etc Enlargement of the prostate and its causes As mentioned above, an excess of mucus in the urine is most commonly due to lesions in this region, and the microscopical examination of the urinary deposit is indispensable In chyluria the obstructed lymphatic ruptures into the bladder or renal pelvis

**Diseases of an ureter or ureters** are uncommon causes of proteinuria, the most frequent among them being stone

**Diseases of a kidney or kidneys** are generally the first to be considered, and sometimes, unfortunately, are thought of as the only cause of proteinuria The renal lesions may be classified under two headings (a) kidney diseases proper, (b) secondary disorders of kidneys

**Kidney Diseases Proper.** Trauma, nephritis, tuberculosis, stone, tumour, and so on The concentration of protein in advanced chronic nephritis may be very small and its detection important, in nephrosis, as a rule, it is high

**Secondary Disorders of Kidneys** Under this heading are included vasomotor and circulatory disturbances in the kidneys themselves Thus "intermittent or benign proteinuria" may be classified here but has been considered in a separate section above, and also 'proteinuria in pregnancy,' many examples of which are due to congestion of the kidneys from pressure by the gravid uterus (see later) Congestion due to a failing heart is in the same category, likewise the circulatory effects in diabetic coma Proteinuria due to surgical shock or to cold baths in certain subjects, comes here too Febrile proteinuria is more difficult to place accurately In fevers there is undoubtedly a complicating nephritis in a proportion of cases and this would of course be classed as "kidney disease proper" But in a much larger proportion of cases the proteinuria is transient and unaccompanied by any clinical signs of renal involvement, some at least of these proteinurias may be regarded as due to renal congestion In a small proportion of cases, and after scarlet fever in particular, it may be difficult to assess the significance of the proteinuria which in some instances persists for years, possibly some of these were present before the fever (see also under "Intermittent Proteinuria") Though febrile proteinuria is often classified together with albuminuria due to poisoning by chemicals, under the heading of "toxic," it seems more logical to separate the chemical poisons and to regard their action as definitely productive of renal damage Experimentally there is clear cut evidence of this and clinically the testing of the urine for protein before and after administration is a practical and useful means of detecting damage to the kidneys by such drugs as the organic arsenicals, mercury, antimony, etc, and such proteinuria is an indication for cessation of treatment In industrial or in deliberate poisoning (arsenic lead, mercury, etc) the proteinuria may be significant,

as when accompanied by clinical signs of kidney damage, or of uncertain import if there are no such signs, and the urine was not known to be free from protein before exposure to the risk

It is generally accepted that ingested proteins are invariably digested and absorbed only as amino acids, and there is no unequivocal evidence that protein by the mouth, however large the amount has any influence on the excretion of protein in the urine of man

Strenuous muscular activity is well recognised as causing or increasing proteinuria intermittently, as in soldiers, athletes, members of rowing crews, etc., but does so only in the minority. Renal congestion may be the explanation, but clearly it is necessary that each case be carefully examined to exclude other and serious causes

This brings us to the practical point of what evidence should be secured before labelling proteinuria "benign". Having made a thorough clinical examination, and having excluded contamination, the fact that the proteinuria is intermittent itself is a strong point in favour of its insignificance, though it has been found that proteinuria may become intermittent in the last stage of a nephritis which is clearing up, particularly after scarlet fever. If it is clearly influenced by posture, that again makes an organic lesion unlikely. An examination of the centrifuged deposit should always be made, all samples containing protein should be so inspected (see Chapter IV). An occasional leucocyte, epithelial cell, hyaline cast or cylindroid may be disregarded but red cells and all other casts should raise the suspicion of the presence of an organic lesion, until repeated and prolonged observations have justified the conclusion that they are immaterial. If it can be shown that the protein is mucus it is not likely to be of importance, but otherwise a study of the nature of the proteins is seldom of practical assistance. Renal efficiency tests (see Chapter V) are of value to exclude gross lesions, but normal findings with these tests by no means indicate that the kidneys are healthy. One or more should be carried out as a safeguard, but the decision as to whether the proteinuria is benign or indicates a mild organic lesion depends on the other evidence considered above.

Proteinuria in pregnancy is very common, and its significance in a particular case often of moment in practice. It has long been recognised that in the great majority of pregnant proteinurics the positive urinary test is of no importance, and is ascribed to congestion of the kidneys due to the pressure of the gravid uterus on the renal vessels. With the modern practice of repeated urinary examinations throughout pregnancy, it has become much easier to assess the significance of the proteinuria, any sudden increase just before term, for instance, and any change in the urinary deposit being revealed.

For a satisfactory study, especially in the last month or so, it is essential to avoid contamination by vaginal discharge by careful cleansing before micturition, catheterisation may be required, but



is usually unnecessary for purely chemical and cytological examinations, and there is no point in running any unnecessary risk of infection

The chief aims of the tests for protein are the detection of nephritis or other organic renal lesion in a woman who has become pregnant, and to obtain warning of the onset of pregnancy toxæmia, and especially of eclampsia

**Proteinuria without Lesions of the Urinary Tract** Under this heading are included the excretion of foreign proteins introduced by injection, proteosuria, Bence-Jones' proteinuria, and so called peptonuria. All have been dealt with in previous sections

## TECHNICAL

### Esbach's Method for Total Proteins in Urine

Take a clean, dry Esbach tube (see Fig. 6). Run in urine exactly up to the mark U. Add Esbach's reagent up to the mark R. Close the mouth of the tube with the rubber bung, invert to mix, and set aside undisturbed for twenty-four hours so that the precipitate may settle by gravity.

The following points must be observed —

(a) If the urine is alkaline, make it acid with 33 per cent acetic acid.

(b) Measure the specific gravity of the urine with a urinometer. If the specific gravity exceeds 1010, dilute the urine with one or more volumes of water, making the necessary correction of the reading obtained next day to allow for the dilution. Thus if the specific gravity is 1016, to 5 c.c. of urine add 5 c.c. of distilled water, mix, and run in the mixture up to the mark U, and add the reagent up to the mark R. Next day note the reading, which multiply by 2. *This dilution is necessary because the protein precipitate will not settle properly if the specific gravity exceeds 1010.*

(c) The markings on the tube represent grammes of dry protein per litre of urine.

(d) The method is of little or no value if the protein is less than 0.05 per cent.

### Aufrecht's Method for Total Proteins

Take a clean, dry Aufrecht tube (see Fig. 6) and run in urine to the mark U and Aufrecht's reagent to the mark R. Close the mouth of the tube with the thumb, invert to mix, and return any fluid adhering to the thumb by drawing it across the mouth of the tube. Place the tube in the centrifuge, and throw down the precipitate by centrifuging for two minutes at 5,000 revolutions per minute, or for two and a half minutes at 3,000 r.p.m., or for three minutes at 2,000 to 2,500 r.p.m. Note the reading at the upper surface of the protein precipitate.

*Notes.* (a) The time prescribed for centrifuging *must* be strictly adhered to; otherwise serious errors will result.

(b) If alkaline, the urine should be made just acid with acetic acid

(c) Dilution of the urine is unnecessary whatever the specific gravity may be

(d) The results are expressed on the tube directly in percentages, i.e., in grammes of dry protein per 100 c.c. of urine

(e) The method is of little or no value for quantities of protein less than 0.1 per cent

(f) The method can obviously be only approximate Unfor-

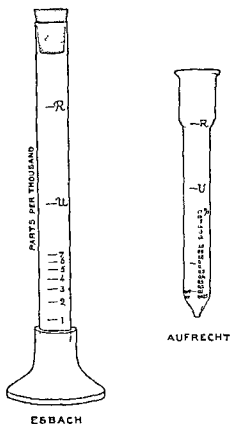


FIG. 6 Esbach's and Aufrecht's tubes

Unfortunately the calibration of the tubes on sale is even worse than that of Esbach's tubes, the chief error being in the calibration of the terminal tapered part, so that two Aufrecht's tubes may give absurdly different results, particularly when the protein is less than 0.1 per cent. The calibrations may be checked in the laboratory by precipitating different dilutions (in physiological saline) of normal blood plasma of which the protein content has been accurately determined by another method.

#### Colorimetric Biuret Method for Total Proteins, Albumin and Globulin

The biuret method of estimating proteins has been known for many years (*cf.* Riegler, Autenrieth), and has been revived by

**Hiller** Pure biuret was used as standard by Hiller, McIntosh and Van Slyke, and suitably diluted serum protein by Fine. In the technique described below the permanent coloured glasses of Messrs The Tintometer Ltd have been utilised instead as standards, and these with the Lovibond comparator<sup>1</sup> (Fig 7) have made the method simple and suitable for routine clinical investigations.

**Principle** The proteins are precipitated by trichloroacetic acid. The precipitate is dissolved in caustic soda solution and copper sulphate is added. The resulting purple solution is centrifuged to throw down the suspended copper hydroxide after which it is matched against the glass standards and the result noted.

In the differential estimations, the globulins are precipitated by half saturation with ammonium sulphate, and the albumin is estimated in the filtrate by the biuret technique, mucus if present, is precipitated with the globulins. Howe's method of fractionation with 22 per cent sodium sulphate may be used instead of the ammonium sulphate, but it is more tedious, and so is not described below (*cf* Remarks).

The glass standards were prepared by matching in the Lovibond comparator a series of solutions of known protein content which had been prepared from normal human serum and treated by the same biuret technique. The protein content of the diluted serum was estimated by the Kjeldahl method, due allowance being made for the non protein nitrogen.

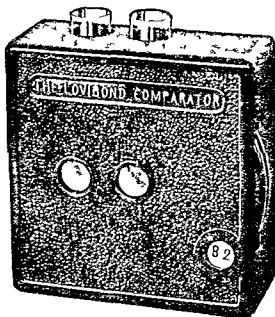


Fig 7 The Lovibond comparator (See also Fig 5<sup>9</sup> p 296)

<sup>1</sup> The apparatus and reagents may be obtained from Messrs The Tintometer Ltd, Milford Salisbury or from Messrs The British Drug Houses Ltd London.

Reagents and Centrifuge Tubes 10 per cent trichloroacetic acid solution

Thirty per cent sodium hydroxide solution

Five per cent copper sulphate solution, 5 gm of crystalline  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  in water to 100 c c

Saturated ammonium sulphate solution, 52.8 gm of A R quality  $(\text{NH}_4)_2\text{SO}_4$  in water to 100 c c If necessary warm slightly to dissolve, and make up to volume at room temperature

Graduated centrifuge tubes preferably should be tapered and of about 15 c c total capacity, graduated from 1 to 10 c c in steps of at least 0.5 c c Though not so satisfactory it is possible to work with non graduated tubes, the volume of each reagent added being accurately measured so that the total volume is as prescribed

**Technique for Total Proteins in Urine** Take the reaction to litmus paper, if acid neutral or very slightly alkaline do not modify it, but if markedly alkaline (e.g., due to gross bacterial decomposition) add glacial acetic acid drop by drop to some 20 to 50 c c urine till the reaction is neutral or slightly acid (this method introduces a negligible dilution as a rule, in exceptional cases a fresh specimen must be substituted, or the dilution must be done quantitatively)

Mix in a graduated centrifuge tube —

Urine	5 c c
10 per cent trichloroacetic acid	5 „

allow to stand for a few minutes until the precipitate clumps Centrifuge thoroughly and decant the supernatant fluid as completely as possible, by inverting the tube carefully and wiping the mouth with filter paper

Add to the precipitate 1 or 2 c c of water and 1 c c of 30 per cent NaOH, shake till the protein has dissolved

Add 1 c c of 5 per cent crystalline copper sulphate solution and water to exactly 10 c c Mix thoroughly for at least one minute and centrifuge well until all the precipitate of cuprous hydroxide has been thrown down

Transfer the clear supernatant fluid to a comparator tube which place in the right hand recess of the comparator Match against the first disc (20 to 180 mgm in steps of 20 mgm per 100 c c) and note the result If the unknown exceeds 180 mgm substitute the second disc (200 to 360 mgm) If the unknown is above 360 repeat the test from the beginning using less urine, read, and multiply by the appropriate factor Thus if 2 c c of urine, with 2 c c of trichloroacetic acid, be employed, multiply by 2.5

**Differential Estimation of Urinary Proteins** The urine must first be made slightly alkaline to litmus, e.g., by adjustment of a relatively large bulk with glacial acetic or 30 to 40 per cent NaOH respectively The correct reaction is pH 7.4 and this may be secured with addition of acid or alkali and phenol red by means of a Lovibond comparator and appropriate pH disc (cf p 295), but

with a little experience litmus paper may be employed instead and satisfactorily

Mix in a boiling tube or flask 10 c c of the adjusted urine and 10 c c of saturated ammonium sulphate (52.8 per cent) Allow to stand till the precipitate of globulin (plus mucus, if present), flocculates, and filter

Mix in a graduated centrifuge tube 5 c c of filtrate and 5 c c of 10 per cent trichloroacetic acid, centrifuge and decant completely Add another 5 c c of filtrate and 5 c c of the acid centrifuge and decant completely Treat the combined precipitate (which is derived from 5 c c of urine) with NaOH and  $\text{CuSO}_4$  as described under "Total Proteins" The reading gives directly the mgm of albumin per 100 c c

Calculate the globulin (or globulin plus mucus) -by difference, total protein—albumin

The above technique will be found satisfactory in the majority of cases, but clearly the details may be modified to suit special circumstances or conditions Thus if a big centrifuge and tube is available 10 c c of filtrate may be treated with 10 c c. of trichloroacetic acid in one stage, if the total protein is high less than or if the proportion of albumin is low, more than 10 c c of filtrate may be better, in which case the appropriate calculating factors must be introduced

**Remarks** It is important that the reagents do not become contaminated by ammonia absorbed from the laboratory atmosphere If they do the final colour will be too blue and too high results will be obtained This is easily checked by putting up a blank from time to time using water instead of urine The blank of course should be colourless

Moreover in the differential estimation it is essential that after precipitation with trichloroacetic acid the supernatant fluid shall be decanted as completely as possible, otherwise sufficient ammonium sulphate is left behind to yield on the subsequent addition of NaOH enough ammonia to keep some of the cuprous hydroxide in solution It has been shown by experiment that if the decantation is carefully done only a very slight plus error is introduced This can be avoided by using sodium sulphate instead but as noted under 'Principle' this is more tedious

### Detection and Separation of Albumin, Globulins and Mucus

**I Proteins Precipitated by Acetic Acid in the Cold** To about 5 c c (1 in column) of urine in a test tube add about 5 c c of distilled water and mix

Place half of the mixture in a second tube and leave it untreated for comparison To the first tube add 33 per cent acetic acid carefully drop by drop till the maximum opalescence has been obtained The cloudiness may be due to mucus or lipid globulin or both The water is added to prevent deposition of uric acid on acidification

Continue the addition of acetic acid and note whether the

opalescence is reduced or remains unaltered Mucus is insoluble, lipoid globulin is soluble in excess of acetic acid

The test may be rendered more satisfactory as follows —

Place 10 c c of urine in each of three 25 c c. measuring cylinders, A, B and C To A add 10 c c of distilled water (non acidified urine) To B add about 8 c c of water, 33 per cent acetic acid drop by drop till maximum opalescence is obtained, and, finally, water to 20 c c (mucus plus lipoid globulin precipitated) To C add 5 c c of water and 5 c c of 33 per cent acetic acid (mucus precipitated) Compare the opacity of C with B, and of both C and B with the non acidified urine A If C and B are equally cloudy the protein precipitated by acetic acid is all mucus If B is more opalescent than C, there is a mixture of the two proteins If B is opalescent, but C is clear, lipoid globulin, but no mucus, is present

In the writer's experience the protein precipitated by acetic acid in the cold is much more often mucus than lipoid globulin, but mixtures are not uncommon

**II Roberts' Test for Lipoid-globulin (Ex-globulin)** Nearly fill a vessel (*e g*, a measuring cylinder or boiling tube) with distilled water, and add the urine drop by drop If lipoid globulin is present in any quantity, around each drop a milky haze is formed When a fair amount of urine has been added the liquid becomes turbid The turbidity disappears at once on very slight acidification with acetic acid This can be demonstrated strikingly by pouring half of the turbid fluid into a second tube, and adding a *single* drop of 33 per cent acetic acid to one of the tubes Mucus is not likely to be precipitated by distilled water, and in any case is insoluble in acetic acid Pseudo globulin is not precipitated

It is wise always to perform Roberts' test after examining for proteins precipitated by acetic acid in the cold by I, since lipoid globulin redissolves in the slightest excess of acetic acid On the other hand, if the acidification in I is carefully done, traces of lipoid globulin may be detected which are not revealed by Roberts' test

**III Separation of Albumin, Globulin and Mucus** If necessary, the urine is centrifuged to remove red blood corpuscles, etc

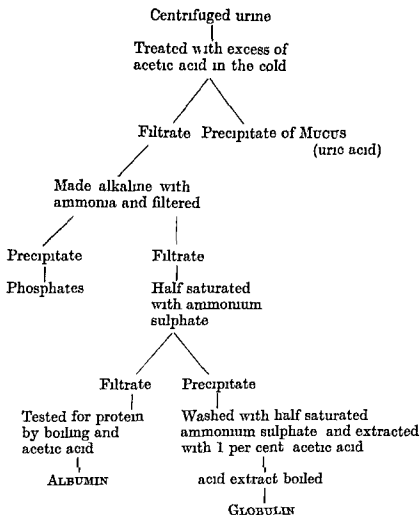
If mucus is absent (see under I) proceed as follows —

Make about 15 c c of urine slightly alkaline with concentrated ammonia solution, stand for a few minutes and filter off phosphates To 10 c c of filtrate add 10 c c of saturated ammonium sulphate solution, and allow the mixture to stand for an hour A precipitate may be globulin, phosphates or ammonium urate Separate the precipitate by centrifuging or filtering and wash it with a mixture of equal parts of water and saturated ammonium sulphate, to remove adherent solution of albumin Treat the washed precipitate with a small volume of 1 per cent acetic acid to dissolve the globulin (and phosphates), filter off uric acid if necessary, and show that globulin is present by the formation of a coagulum on boiling

The supernatant fluid or filtrate removed from the precipitate of globulin is tested for albumin by the boiling and acetic acid test

If mucus is present (see I) proceed as follows —

To about 15 c c of urine add drop by drop 33 per cent acetic acid (in the cold) till acid, and until any precipitate of lipoid globulin has redissolved (1 to 5 c c of acetic acid) Stand a few minutes and filter Make the filtrate slightly alkaline with concentrated ammonia solution, filter off phosphates if necessary, and continue as described above to test for globulin and albumin



#### Detection of Bence-Jones' Protein

Bradshaw's is useful as a preliminary test in routine work. If it is positive the heat test described below should be carried out to establish with certainty the presence of Bence Jones protein if it is negative further investigation is unnecessary. It must be remembered however that Bradshaw's is by no means a specific test, any pathological urine containing an abundance of protein will give a positive result, though commonly only a slight haze

Normal blood or serum, added to normal urine (e.g., 1 to 100) will give a positive reaction, and a similar result is obtained in hæmaturia if of a grade evident to the unaided eye, possibly it is the globulin rather than the albumin fraction which is precipitated by concentrated hydrochloric acid. Urine containing uroselectan will give a false positive reaction (see p 232)

**Bradshaw's (HCl) Test** Perform a ring test, superimposing a few cubic centimetres of the urine on about 5 c.c. of concentrated hydrochloric acid. Bence-Jones' protein gives a heavy curdy precipitate at the junction of the two fluids. A positive test is obtained after considerable dilution of the urine (e.g., 1 of urine to 10 or 20 parts of distilled water), unless the concentration of the protein is very low.

In Bence Jones' proteinuria positive reactions are obtained with all the routine qualitative tests for protein, though a difference in behaviour may be noted with the boiling and acetic acid test, a precipitate occurring earlier than usual in the heating process and redissolving partially or completely on boiling. It is possible even to miss protein altogether by heating rapidly to boiling point.

Resolution on boiling may be demonstrated clearly as follows —

**Two-tube Test** Place about 10 c.c. of slightly acid urine in a test tube and warm to about 60° C — until it is just not too hot to grip firmly in the hand. Invert the tube once or twice to insure an even temperature and suspension of the precipitated protein, and pour half into another tube. Leave one tube for comparison, and heat the other to boiling. Resolution and the degree thereof will be evident on immediate comparison of the opacities of the two tubes.

In theory Bence-Jones' protein is readily detected owing to its precipitation at low temperatures (40° to 60° C), its disappearance on boiling and its reappearance on cooling. In practice the presence of the protein is easily missed, owing to the great influence of the reaction of the urine on its behaviour, particularly when the concentration of the protein is low.

Solutions of the separated protein are affected not only by the reaction, but also by the quantity of neutral salts and the valency of their kations and anions (Hopkins and Savory), but so far as the writer is aware there is generally little or nothing to be gained by adding neutral salts when testing urines for the protein.

If the original urine is alkaline it should be made neutral or very slightly acid to litmus with acetic acid to avoid a precipitate of phosphates on heating. If an excess of acid is added, however, the protein may not come down at any temperature, indeed, over acidification is probably the chief cause of failure to detect the protein by the heat test. With lesser excesses of acid the precipitate may not occur below 60° C, or it may not disappear on boiling. Conversely a slight excess of acid may be necessary to cause the maximum reappearance of the protein precipitate on cooling. A simple method, by which the above possibilities of error are generally avoided, is as follows —



**Heat Test for Bence-Jones' Protein.** Clear the urine by filtering, repeatedly if necessary. Note the reaction to litmus of the filtrate. If alkaline, make it neutral or very faintly acid by the cautious addition of acetic acid (33 per cent.). Place about 5 c.c. of the filtrate in each of three test-tubes. To the first tube add nothing, to the second and third tubes add 1 and 2 drops of 33 per cent. acetic acid respectively. Place the three tubes and a thermometer in a beaker of water. Heat the beaker cautiously so as to raise the temperature slowly, rotating the tubes and thermometer so as to stir the water well, thereby keeping the temperature of the water and of the urines uniform. Observe the changes which occur in each of the tubes and the temperature at which the changes occur. When Bence-Jones' protein is present the urine suddenly becomes turbid at a temperature below  $60^{\circ}\text{C}$ —often between  $40^{\circ}$  and  $50^{\circ}\text{C}$ . After a further rise of a few degrees (but still below  $60^{\circ}\text{C}$ ) the precipitate becomes flocculent. Ordinary plasma proteins (albumin and/or globulin) do not come down till the temperature exceeds  $60^{\circ}\text{C}$ , though in their case also the reaction of the urine affects the temperature at which they are first precipitated.

On raising the temperature to  $100^{\circ}\text{C}$ , Bence-Jones' protein may or may not completely disappear, but when thoroughly boiled in the flame, one of the three tubes will generally show its complete disappearance (Remember, however, that proteins other than Bence-Jones' may also be present) Therefore, after observing the flocculation below  $60^{\circ}\text{C}$ , remove the three tubes from the beaker, and boil each thoroughly in the flame.

On cooling, the protein in the first tube, to which acid has not been added, may not come down so well as the protein in one of the other two tubes, but from the behaviour of the contents of the three tubes a composite picture is obtained which shows the typical behaviour of Bence-Jones' protein.

When the conditions are not correct for complete resolution at boiling-point, it will often be observed that the protein forms a peculiar stringy, sticky coagulum. Bence-Jones' protein may be estimated in urine by the biuret method given above, the writer having found that its biuret equivalent is the same as that of serum proteins, it is precipitated with globulins.

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## CHAPTER IV

### URINARY DEPOSITS: CALCULI AND CONCRETIONS

**Books** Most manuals of clinical laboratory methods give descriptions of urinary deposits. Much detail, with numerous illustrations will be found in *Atlas of Urinary Sediments*, by Reider, Moore and Delepine, 1899.

For a full account of "Cystinuria" the reader is referred to Garrod's *Inborn Errors of Metabolism*.

Accounts of the properties and reactions of cystine, tyrosine and leucine are scattered through text books of physiological chemistry, etc., e.g., those by Cole, Hammarsten and Hedin, Hawk and Bergeim, Plimmer, Emerson's *Clinical Diagnosis* and *Clinical Diagnosis* by Von Jaksch, translated by Garrod.

For full descriptions of urinary calculi see Swift Joly's *Stone and Calculous Disease of the Urinary Organs*, and Winsbury White's *Stone in the Urinary Tract*.

The schemes for analysis of calculi given in most books (e.g., by Hutchison and Hunter, Hawk and Bergeim, Hammarsten and Hedin) are unnecessarily elaborate for purely clinical purposes.

For a fuller account of concretions see Wells' *Chemical Pathology*.

### PRELIMINARY TESTS ON URINARY DEPOSITS

MUCH may be learnt by inspection and a few simple chemical tests.

First the reaction of the urine to litmus should be taken. As a general rule phosphates are deposited only when the urine is alkaline, and urates or uric acid when it is acid. To each of these statements, however, there is an exception. Calcium hydrogen phosphate (stellar phosphate) may be deposited in a urine which is slightly acid, and ammonium urate may separate out in an alkaline urine. Calcium oxalate may be deposited when the reaction is acid, neutral, or alkaline. It is all important to remember that a deposit of phosphates or of urates in a specimen which has cooled down to room temperature is not necessarily abnormal, in fact, in most cases the observation is of no significance. The important points to determine are (a) whether the substance actually is passed *in suspension*, and (b) whether the day's output is excessive. The existence of (a) should only be recorded if the doctor has actually witnessed the micturition. Such a condition may be of significance and require treatment. But by far the commonest cause of so called 'lithuria' is the fall in temperature of the urine from 37° C to room temperature and by far the commonest cause of so called 'phosphaturia' is a shift of the reaction of the urine *after* it has been passed to the alkaline side, due to

bacterial decomposition The existence of (b) can only be decided from a knowledge of the intake and output of the substance

Secondly, if the urine is alkaline, the effect of adding acetic acid (33 per cent) till the reaction becomes acid should be noted Phosphates will redissolve If, on addition of acid, gas is evolved, it will probably be  $\text{CO}_2$  liberated from bicarbonates or carbonates The commonest cause is the bacterial decomposition of urine which has been allowed to stand, whereby urea is converted into ammonium carbonate Bicarbonates may be excreted in fresh urine in amounts sufficient to give this reaction, either when the urine is alkaline naturally (normal "alkaline tide," or diet rich in alkali or substances which yield alkali in the body), or as a result of the administration of certain drugs, *e.g.*, sodium bicarbonate, potassium citrate, etc If the urine is acid, dilute hydrochloric acid (10 per cent v/v) should be added Calcium oxalate will go into solution (Calcium oxalate is insoluble in acetic acid and in weakly acid urine, but is soluble in hydrochloric acid Uric acid and urates are insoluble in dilute hydrochloric acid)

Thirdly, in the case of acid urine, the effect of warming should be tried The urine should be well shaken and a sample carefully warmed in a test tube to about  $60^\circ\text{C}$  (not too hot to hold), when uric acid or urates will generally go back into solution It must not be boiled lest phosphates or protein be thrown out of solution If samples of the supernatant clear urine have already been removed, the deposit will be more concentrated than originally, in which case warming may not cause a re solution of urates or of uric acid

Fourthly, it should be noted whether or no the deposit is pigmented Bile or blood may cause a deposit to be coloured Uric acid and urates when deposited may carry down with them urinary pigments Thus the colour of a "cayenne pepper" deposit is due to urochrome and uroerythrin which have been carried down with uric acid crystals But the writer would warn the reader that a uratic deposit occasionally may be white, or only very faintly stained with urochrome, students not uncommonly err in believing that uric acid deposits are *invariably* pigmented

Lastly, it is essential to bear in mind that deposits are most often of a mixed nature, *e.g.*, leucocytes and bacteria, cells and crystals, etc It follows, therefore, that one or more of the above tests may not be conclusive In fact, *it is not safe to give any opinion on a deposit without making a microscopic examination* The above tests are often of value as a preliminary, or as an adjunct to microscopic examination

## THE MICROSCOPICAL EXAMINATION OF URINARY DEPOSITS

No apology is needed for including a brief account of the microscopical examination of urinary deposits in a book on chemical tests The chemical pathologist should not hand over the examination of the deposit from a urine in which he has found

protein, to his colleague, the clinical pathologist. He should examine it himself in an endeavour to discover the cause of the proteinuria.

The preparation of deposits for examination is made in one of three ways —

- (a) The deposit is allowed to settle by gravity, for preference in a conical receiver
- (b) The urine is thoroughly mixed to obtain an even suspension, and a drop of the suspension is examined
- (c) The urine is centrifuged

Method (a) is sufficient in order to observe the naked eye appearance of a deposit, and in certain circumstances it is the only method available in clinical work. Whenever possible, however, the urine should be centrifuged, unless there is a gross deposit, when it is, of course, unnecessary.

Method (b) is of value, for instance, as a preliminary to counting the number of cells passed in a given specimen. Thus it is sometimes employed in following the progress of a case of pyelitis. The urine is well mixed, and a drop mounted. Either the number of cells per field may be counted and the mean in a number of fields calculated, or the number per cubic millimetre of urine may be determined with the aid of a blood counting chamber. Addison has made interesting observations by counting the number of cells and casts in the night's urine (twelve hours), both in health and disease, but this is not practicable in routine work.

Method (c) is generally the method of choice.

### THE NATURE OF URINARY DEPOSITS

In the table on p. 47 will be found a fairly complete list of the "organised" and "unorganised" substances which occur as deposits or float on the surface of urine. As an aid to memory the following may be convenient.

Deposits may be classified under *three* headings, each beginning with 'C', viz. "Cells," "Casts," and "Crystals," and a fourth group viz., "Miscellaneous." The heading "Crystals" should in full be "Crystals and amorphous chemical deposits." In the first group are included *three* types of cells, viz., white blood corpuscles, red blood corpuscles, and epithelial cells. Likewise casts are commonly of *three* types, viz., hyaline, granular and cellular. Cellular casts may be coated with either epithelial cells (epithelial casts) or leucocytes, or red blood corpuscles (blood casts). Some times casts are coated with fatty granules (fatty casts). Should these fatty granules be doubly refracting when examined under crossed Nicol's prisms, myelin kidney should be suspected. Lastly, there is an uncommon type of cast, the "waxy" cast. This has nothing to do with waxy or amyloid disease. "Cylindroids" are long cast like structures which have no pathological significance. The commoner crystals and amorphous chemical deposits similarly fall into *three* main groups, viz., phosphates, urates (including

## Urinary Deposits

- (1) CELLS
- (2) CASTS
- (3) CRYSTALS AND AMORPHOUS CHEMICAL DEPOSITS
- (4) MISCELLANEOUS

## (1) CELLS

Leucocytes  
Erythrocytes  
Epithelial cells

## (2) CASTS

Hyaline  
Granular  
Epithelial  
Leucocyte  
Blood (r b c) } Cellular  
Fatty  
Waxy  
Cylindroids

## (3) CRYSTALS AND AMORPHOUS CHEMICAL DEPOSITS

*Acid Urine**Alkaline Urine*

Uric acid

Urates { Crystalline Sodium  
          { Amorphous { Potassium  
                          { Sodium  
                          { Ammonium

Ammonium urate (crystalline)

Calcium oxalate

Calcium oxalate

Calcium hydrogen phosphate (stellar  
phosphate) in faintly acid or neutral  
urines

Calcium hydrogen phosphate (stellar)  
Ammonium magnesium phosphate  
(triple)

Bilirubin (hematoidin)

Amorphous phosphates { Calcium  
                                  { Magnesium  
Amorphous carbonates

Cystine

Calcium carbonate

Xanthine

Cholesterol

Tyrosine

Leucine

Hippuric acid

Indigo blue

Indigo blue

## (4) MISCELLANEOUS

Mucus

Spermatozoa

Bacteria and yeasts

Parasites { *Bilharzia*  
              { *Filaria*  
              { *Echinococcus*

Starch granules from dusting powders

Foreign

bodies

{ *Hairs*  
Cotton wool  
Tow

Only

substances

{ True lipuria (see Chapter XIV)  
Chyluria (see Chapter XIV)  
Lubricants from catheters  
Toluene as preservative (in suspension and on surface)  
Chloroform as preservative (in suspension and at bottom)  
Liquid paraffin (contamination from rectum)  
Milk (added by patient)

hydrogen urate or uric acid) and calcium oxalate. Deposits of phosphates and of urates may be either amorphous or crystalline. Calcium oxalate is always deposited in crystalline form. Rarely certain other crystals may be found viz, cystine, tyrosine, leucine, bilirubin, indigo blue, cholesterol, hippuric acid and calcium carbonate. A deposit of xanthine has only once been described

(Bence Jones) Sometimes, after large doses of alkali, the volume of the phosphate deposit which separates after the urine has stood some hours is truly astounding. The group of "miscellaneous substances" includes the mucus which commonly separates as a light flocculent cloud in urine which has been allowed to stand. It is frequently observed thus in urines obtained from perfectly healthy individuals. It is derived from the cells forming the mucous membrane of the urogenital tract. In certain pathological states, particularly cystitis, there may be an excess of mucus secreted, which separates as a glairy stringy mass at the bottom of the urine glass. The remaining deposits may be classified under the headings of spermatozoa, bacteria and yeasts, parasites (bilharzia, filaria, and echinococcus), foreign bodies, and oily substances.

It is obviously outside the scope of this book to deal with most of these. The reader is referred to the list of books at the beginning of this chapter. A few points of chemical interest, together with some of the difficulties brought to the writer by students, will be noted *seriatim*.

### Cells and Casts

In the writer's experience, the beginner often has considerable difficulty in differentiating one type of cell from another. The table below and the Fig on p. 49 may be helpful in combination with the usual account given in standard books.

	R.B.C	W.B.C	Ep.C
Size . . .	Average, $7\mu$ . Varies considerably with tonicity of urine	7 to $10\mu$ . Slightly larger than average R.B.C	Varies with type. Some slightly larger, most much larger than W.B.C
Colour	Very pale yellow	Colourless	Colourless
Appearance	Homogeneous Not granular May be crenated Biconcave discs Double ring contour Not nucleated	Heterogeneous Granular  Spherical  Nucleated	Heterogeneous Often granular  Of various shapes  Nucleated

In hypertonic urines red blood corpuscles are smaller than on the average. In hypotonic urines the red blood corpuscles are larger than the average, sometimes they are so swollen as to be practically spherical and lose their double contour. Crenation, though most commonly found when the urine is hypertonic, may also occur in hypotonic solutions. Ponder and Saslow suggest that crenation is due to a failure of the red cell to maintain its peculiar shape.

It is a good rule not to label any object a cast unless it has parallel edges. The beginner is most likely to mistake for a cast either a hair, a scratch in the slide or coverslip, or a degenerated

squamous epithelial cell. The accompanying diagram (Fig. 8) may assist him. The secret in hunting for casts is to focus the condenser

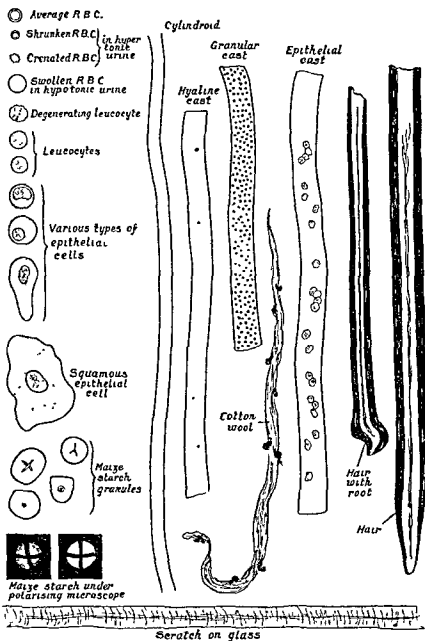


FIG. 8. Diagram to assist in the identification of cells, casts, and starch granules. All are drawn to the same scale, using a micrometer eyepiece.

(p. 7), and to cut down the light to a minimum by means of the diaphragm.

### Uric Acid and Urates

Clinically a deposit of uric acid or urates is often referred to as lithuria. As previously mentioned, it is most often caused by cooling



of the urine *in vitro*, and only occurs at acid reaction (ammonium urate excepted) Uric acid crystallises in many different forms (barrels, plates, prisms, needles, sheaves, rosettes, hexagons, etc (Fig 9)), it is generally, but not invariably, coloured by adsorbed

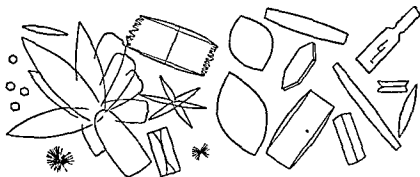


FIG 9 Different forms of uric acid

urinary pigments, and redissolves on heating to about  $60^{\circ}\text{C}$  (cf p 45), or in excess of alkali

Urates are often amorphous, but sodium urate (thorn apple crystals) and ammonium urate (pigmented spheres or "thorn apples without the thorns," and thorn apple crystals) may be crystalline (Fig 10) Ammonium urate occurs at alkaline reaction



FIG 10 Uratic deposits (a) Amorphous urates (b) Pigmented spheres of ammonium urate (alkaline urine) (c) Thorn apple crystals of urates of ammonium or sodium

Like uric acid, urates are usually pigmented, and redissolve on warming or in excess of alkali

It is useful to know the morphological appearance of these deposits in order to be able to differentiate them from other substances but it must be admitted that they are of very little practical importance It is only when they occur in suspension *in vivo* that they can be of any clinical significance, because they may cause irritation in the urinary passages, though that is exceptional, similarly the passage of uric acid and urates in suspension in the urine is seldom accompanied by the formation of calculi and it is certainly unsafe to regard lithuria as evidence of the presence of stones, though it may lead to a suspicion of their existence

### Phosphates

Reference has been made (p 44) to the importance of reaction and of bacterial decomposition of urine, in the production of a

deposit of earthy phosphates True phosphaturia is much commoner than true lithuria, but the former, like the latter, is seldom accompanied by calculus When the phosphaturia (ammonium magnesium phosphate) is due to bacterial decomposition *in vivo* in the bladder (cystitis, prostatitis), it is of more serious significance, and is more likely to be associated with the formation of stone Many subjects are needlessly alarmed by the passage of a cloudy urine due to a suspension of phosphates, clinically such a condition is noted most frequently in association with wasting, worry, serious mental depression, or severe mental work or strain (Langdon Brown and Hilton) Crystals of calcium hydrogen (stellar) phosphate are uncommon, whereas those of ammonium magnesium (triple) phosphate are often seen in urinary deposits, stellar phosphates occur as clusters or rosettes of prismatic or simple needles (Fig 11), triple phosphates as "coffin lids" or "knife rests," or as feathery crystals (Fig 12)

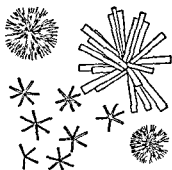


FIG 11 Stellar phosphates (calcium hydrogen phosphate)

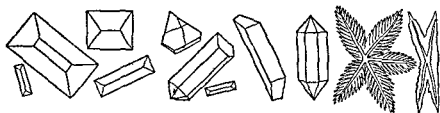


FIG 12 Triple phosphate crystals (ammonium magnesium phosphate)

### Calcium Oxalate

Morphologically the commonest form of calcium oxalate is the flattened octahedron or "envelope" crystal, though it may appear in the shape of a biscuit or dumb bell (Fig 13) That it may be deposited

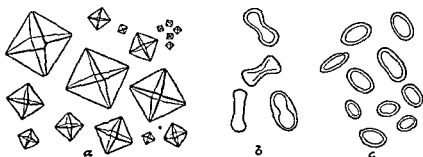


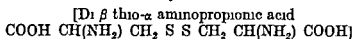
FIG 13 Calcium oxalate crystals

- (a) Octahedra or 'envelope' crystals
- (b) Dumb bells
- (c) Biscuit forms

- at any reaction, is insoluble in acetic but soluble in mineral acids, has previously been noted (p 45) The crystals are most commonly deposited after the urine has been cooled *in vitro*, but they may be present *in vivo* in suspension in the urine and cause irritation, and occasionally even slight hæmaturia As in the case of phosphates and urates, it is only in a very small minority of instances that the deposit is accompanied by the formation of a calculus

Many vegetable foods contain oxalates, but Langdon Brown and Hilton consider that strawberries, spinach and rhubarb are most likely to produce oxaluria with symptoms, a second factor they mention is the formation of oxalic acid from sugar by fermentation in the stomach

### Cystine



The recognition of cystine crystals is important, because their discovery is essential to establish the diagnosis of cystinuria, unless a cystine calculus has already been removed from the subject and its identity has been settled The condition is rare and is due in most instances to an inborn error of metabolism The cystine may be derived from both endogenous and exogenous metabolism The condition is serious because of the liability of the formation of calculi

The colourless crystals (Fig 14) are hexagonal in shape and are soluble in alkalis especially ammonia, and in mineral acids They are insoluble in water, acetone, alcohol, ether and acetic acid It is

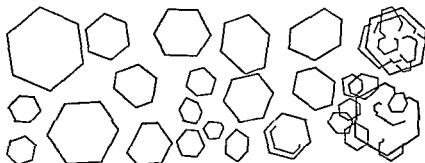


FIG 14 Hexagons of cystine

essential that the urine be acid before examining the deposit for cystine crystals Neglect of this precaution has no doubt in part been responsible for some of the negative findings in known cases of cystinuria When the urine is alkaline, it should be acidified with acetic acid allowed to stand for half an hour or longer, and then centrifuged In some cases, when the concentration of cystine is low, even acidification will not precipitate it Gaskell's method (see below) should then be applied or the cystine may be estimated (*e g*, by the methods of Looney or of Medes)

Certain forms of uric acid are very like cystine, but the former are nearly always tinted by urochrome or other pigments, whereas cystine crystals are invariably colourless. Both are deposited in acid urines, and so a mixture of cystine and uric acid crystals may occur. Usually uric acid crystallises out in more than one form. Even if the crystals are all colourless typical hexagons it is advisable to perform confirmatory tests before diagnosing cystinuria. Uric acid crystals are readily differentiated owing to the fact that they do not dissolve in dilute mineral acids, whereas cystine crystals do.

Iodoform crystals are hexagonal in shape, but pale yellow in colour, and on rare occasions might gain access to the urine (*e.g.*, from surgical dressings) and so cause confusion. Iodoform could be distinguished by its solubility in acetone or ether.

The amount of cystine in urine may be determined gravimetrically by Gaskell's method, or colorimetrically, *e.g.*, by the methods of Looney or of Medes. The daily output of cystine by a cystinuric on a mixed diet is commonly from 0.3 to 0.5 gm. Normal urine may contain traces—0 to 10 mgm per 100 c.c. (Looney), 1 to 84 mgm per diem (Medes)—but never sufficient to crystallise out.

**Identification of Crystals as Cystine.** Separate the suspected crystals by centrifuging. Remove the supernatant fluid and mount a drop of the deposit. Focus the crystals and add a drop or two of 30 per cent v/v hydrochloric acid. The cystine crystals dissolve rapidly, uric acid crystals are not affected. The process of solution may easily be watched under the microscope. Other mineral acids (*e.g.*, 20 per cent v/v  $\text{H}_2\text{SO}_4$ , phosphoric acid S.G. 1.75, diluted 1 in 2) give a similar result.

**Test for Cystine in Solution in Urine.** Fill a test tube half full of urine and boil with 2 or 3 c.c. of 40 per cent sodium hydroxide. Add 3 or 4 drops of a saturated lead acetate solution, when a black precipitate of lead sulphide will form if cystine be present. This test is not conclusive, however, unless the urine be free from protein because in the protein are included sulphur containing amino acids from which sodium sulphide is formed by the treatment with strong alkali.

**Gaskell's Acetone Method as a Means of Separating Cystine Crystals.** Make 500 c.c. of urine alkaline to litmus with concentrated ammonia solution, and add a saturated solution of calcium chloride until there is no further precipitate of oxalates and phosphates (test small portions after each addition of the calcium chloride, by filtering or centrifuging, and adding a drop of the calcium chloride solution to the filtrate). Add 500 c.c. of acetone, mix, allow to stand for a few minutes, and filter. Acidify the filtrate with acetic acid and leave it undisturbed overnight or for longer. Carefully decant most of the supernatant fluid, and transfer the remaining fluid and all the precipitate to a centrifuge tube. Centrifuge, and decant as completely as possible. Dissolve the precipitate in about 5 c.c. of 10 per cent v/v HCl, clear by centrifuging, and transfer the supernatant fluid to another tube. Make slightly alkaline with

ammonia, and then acid with acetic acid. Cool and start the crystallisation, if necessary, by adding acetone a few drops at a time, but do not use more than an equal volume in all. Examine the deposit under the microscope.

Sometimes the cystine comes out as tufts of needles, which may be converted into the typical hexagons by re solution in 10 per cent v/v HCl, followed by the treatment with ammonia, acetic acid and acetone as before. Cystinuria should not be diagnosed until the characteristic hexagons have been obtained.

### Tyrosine

[ $\beta$  parahydroxyphenyl  $\alpha$  aminopropionic acid  
 $C_6H_4(OH)CH_2CH(NH_2)COOH$ ]

Pure tyrosine crystallises in sheaves which are colourless. Deposits of tyrosine in urine also occur in the form of sheaves (Fig 15).

The crystals, however, are frequently stained yellow, owing to the associated jaundice which is found in most of the diseases in which tyrosinuria occurs.

Tyrosine is very slightly soluble in cold, more soluble in hot water. It dissolves easily in dilute mineral acids and alkalis. To be precipitated on neutralising the solution, it is insoluble in acetic acid, acetone, alcohol and ether. It gives Millon's reaction.

Tyrosine is an uncommon deposit in urine, and has been reported most

often, together with leucine, in acute yellow atrophy of the liver. It may be found apart from leucine. It has also been observed in certain cases of acute phosphorus poisoning, cirrhosis of the liver and leukaemia. In fact, it may appear in any condition in which there is a great breaking down of body tissue.

Certain substances occurring in urinary deposits are apt to be mistaken for tyrosine, particularly when the urine contains sufficient bile to tinge the deposit yellow. Thus stellar phosphates, certain forms of uric acid and one form of bilirubin (haematoidin) crystals may cause confusion. Indeed, many of the older reports are of little value, since they were limited to the microscopical appearance.

If a polarising microscope is available tyrosine sheaves which are anisotropic may readily be distinguished from stellar phosphates which are isotropic. On addition of alkali tyrosine crystals dissolve. Stellar phosphates do not. On addition of acetic acid the converse holds. Uric acid crystals are anisotropic, but are insoluble in hydrochloric acid. Bilirubin crystals may be differentiated by their solubility in acetone.

**Identification of Crystals as Tyrosine** Make the urine slightly

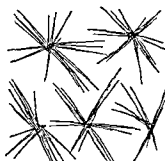
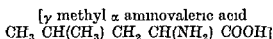


FIG 15 Tyrosine

acid with acetic acid, separate the suspected crystals by centrifuging, and decant the supernatant fluid. Wash the residue in the centrifuge tube with acetic acid (33 per cent), centrifuge and reject the supernatant fluid. This will get rid of phosphates. Shake the residue with a few cubic centimetres of acetone, spin, and reject the supernatant fluid. This will remove crystals of bilirubin. Mount a drop of the deposit, and observe under the microscope the result of adding a drop or two of hydrochloric acid (10 to 30 per cent v/v). Uric acid crystals will be untouched, tyrosine sheaves will dissolve.

### Leucine



Leucine occurs as a rare deposit in urine in the form of yellow spherical masses (Fig 16). It is usually, if not always associated with tyrosine. It has been reported most often in acute yellow atrophy of the liver, but may also occur in certain other pathological



FIG 16 Leucine (After Hutchison and Rainy)

conditions associated with tyrosine (see under Tyrosine). It has the same significance as tyrosine, pointing to extensive destruction of tissue within the body.

The balls of leucine as seen in urine are impure leucine. (Pure leucine crystallises in thin white hexagonal plates.) Leucine is soluble in water, alkalis and acids, including acetic acid, but only slightly soluble in alcohol. It is insoluble in acetone and ether. Certain forms of urates are the substances most likely to be mistaken for leucine, in fact, it is probable that most of the deposits recorded as leucine were actually urates.

**Identification of Crystals as Leucine.** Separate the suspected crystals by centrifuging and mount a drop of the deposit. Focus a bunch of the crystals and note the effect of adding a drop of 33 per cent acetic acid or of 30 per cent v/v hydrochloric acid. Leucine crystals dissolve rapidly, urates do not, but may give place to uric acid crystals.

### Bilirubin (Hæmatoidin), $C_{33}H_{36}N_4O_6$

Bilirubin may be deposited in any acid urine containing much bile pigment, either in the amorphous form or as tufts of short brown needles, or as reddish brown cubes and rhombic plates (Fig 17) It



FIG 17 Bilirubin

- (a) Amorphous (c) Rhombic plates  
(b) Tufts of needles (d) Cubes

is very apt to stain cells, crystals, and other material in the deposit. It stains uric acid crystals deeply, and may modify their shapes. Crystals of bilirubin are easily recognised by their solubility in acetone (see under Tyrosine).

The following table summarises the solubilities of the deposits mentioned above

Urinary Deposit	SOLUBILITY IN					
	Alkalies	Dilute Mineral Acids	Acetic Acid.	Alcohol	Acetone	Ether
Uric acid and urates	Sol	Insol	Insol	Insol	Insol	Insol
Earthy phosphates	Insol	Sol	Sol	Insol	Insol	Insol
Calcium oxalate	Insol	Sol	Insol	Insol	Insol	Insol
Cystine	Sol	Sol	Insol	Insol	Insol	Insol
Tyrosine	Sol	Sol	Insol	Insol	Insol	Insol
Leucine	Sol	Sol	Sol	Slightly sol	Insol	Insol
Bilirubin	Sol	Sol	Sol	Slightly sol	Sol	Slightly sol

### Indigo Blue, $C_{16}H_{10}N_2O_2$

Indigo blue is derived from indican (potassium indoxyl sulphate). It is a rare deposit, and is only formed after the urine has stood. Potassium indoxyl sulphate yields free indoxyl in acid solution, and this is oxidised by air to indigo blue. The occurrence of indigo in acid urines might thus be accounted for, but, though it has been found in acid urine, it is much more commonly deposited in urines which have become alkaline owing to ammoniacal decomposition. It has been suggested that in these alkaline urines the indigo is

formed by the decomposition of indoxyl glycuronates, which are less stable than the sulphates, but the writer has found when attempting to isolate indican that it is very liable to yield indigo blue if ammonia is present. The conditions required for the formation of indigo in stale urine are not known, the concentration of indican seems of little importance.

The presence of indigo may be suspected from the bluish tinge of the urine or of the deposit. Sometimes it forms a bluish pellicle on the surface of the urine. It tends to stain other crystals in the deposit, and so may appear to be deposited in varying forms. True indigo in urine may be amorphous (Fig 18, a) or in the form of blue needles in tufts (Fig 18, b). When crystallised slowly from chloroform it may come out as blue rectangular plates (Fig 18, c). Indigo blue is insoluble in water, very slightly soluble in alcohol, and easily soluble in chloroform. Its solutions give an absorption band in the red (Fig 48, facing p 220).



FIG 18 Indigo blue

- (a) Amorphous blue particles  
(b) Tufts of blue needles  
(c) Blue rectangular plates  
(from chloroform)

#### Cholesterol, $C_{27}H_{45}OH$

Cholesterol crystals are very rare in urine, and are probably in all cases derived from cystic cavities communicating with the

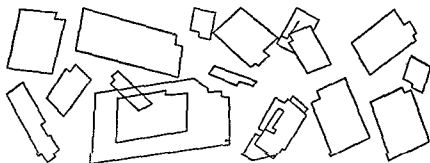


FIG 19 Notched plates of cholesterol,  $C_{27}H_{45}OH \cdot H_2O$

urinary tract, or from "cystic swellings" formed in the tract, *eg*, pyonephrosis. They are easily recognised by their shape—rectangular plates with a notch at one corner (Fig 19)—and by their ready solubility in chloroform.

#### Xanthine (Dioxypurine) and Hippuric Acid (Benzoyl glycine)

Crystals of these substances are extremely rare, wherefore the reader is referred to larger works. Crystals of hippuric acid are of no clinical significance.

#### Starch Granules

Starch granules in the urinary deposit not infrequently present difficulties. In an ordinary unstained wet preparation they may



often be recognised by the concentric arrangement of the laminae of which each is composed (potato starch). In some varieties of starch (maize) this laminated appearance is not obvious, but in the centre of each granule may be seen a "hole" or a "split". The appearance under the polarising microscope is characteristic, the bright or doubly refractile portion of each granule closely resembling a Maltese cross (maize starch) (Fig 8 on p 49). It is easy to establish the identity of starch granules by mounting a second drop of the deposit in a solution of iodine (*e.g.*, Lugol's solution). By this means starch granules are stained a deep blue or bluish black.

Starch gains access to the urine from dusting powders, and is very commonly seen in the urines of infants and young children, less commonly in the urines of adults, and then more often in women's than men's.

### Note on the Contamination of Urine by Fæces

Microscopical examination of the centrifuged deposit may reveal substances which can only have been derived from the fæces. In this way faecal contamination may be detected in urines which to the naked eye appear quite normal. Such contamination is, of course, more likely to occur in females than in males. In fact, in these cases it is almost safe to diagnose that the particular urine was passed by a female. Cellulose remains and undigested or partially digested muscle fibres (see Chapter XXIV) are examples of deposits derived from fæces. On two occasions the writer has been surprised to see ova of *Trichocephalus dispar* in the deposits of girls' urines. Catheter specimens of urine from these patients were free from ova. The fæces contained large numbers.

### URINARY CALCULI

Urinary calculi are composed of urinary crystalloids and a little colloid material (mucus and blood proteins), which acts as a binding substance. The main crystalloids are nearly always urates (including hydrogen urate or uric acid) or phosphates or calcium oxalate. Mixtures of two of these groups are common, and occasionally a mixture of all three occurs. The table below, compiled from the writer's personal analyses illustrates this statement.

Calculi Removed From	Phos phates	Urates	Calcium Oxalate	Phos phates and Urates	Phos phates and Calcium Oxalate	Calcium Oxalate and Urates	Calcium Oxalate Urates and Phos phates
Kidneys	10	0	0	0	19	1	0
Bladder	4	1	0	7	5	1	1
Total	14	1	0	7	24	2	1

The total number of calculi, viz, forty nine, is so small that it is not claimed that these figures represent the true average distribution among the different groups. It would, of course, be necessary to collect many hundreds of analyses for this purpose (*cf* Nakano). Nevertheless, one or two interesting points emerge. First, considerably more than half, actually thirty four out of forty nine, of the stones analysed were mixtures and not "pure" stones. It should be added that in most cases there was a large quantity of each of the two groups of substances composing the calculus, and not merely a slight trace of one group. The reader may wonder why, in calculi, phosphates may be associated with calcium oxalate or with urates, whereas in urinary deposits the reaction of the urine rarely permits such mixtures (see list of urinary deposits). It should be remembered, first that the reaction of the urine in the kidneys or bladder varies from time to time, and, secondly, that calculi do not redissolve since the colloidal precipitate which forms an essential part of each calculus is "irreversible" (*cf* Joly). Incidentally, when determining the composition of a calculus, traces of protein or "albuminous matter" will always be noted (not included in the above table). In fact, when analysing a supposed stone or "gravel" the absence of any "albuminous matter" is very suggestive of deception or error on the part of the patient (see later under Concretions).

Secondly, the above results support the work of Benjamin Moore and of others who have pointed out that many text books are in error in stating that calculi are most commonly composed of uric acid or urates.

Thirdly, the frequency of mixtures accounts for one of the difficulties in treatment. Thus, if alkalis are administered there is a risk of encouraging the deposition of phosphates. Conversely, if acid sodium phosphate is prescribed, uric acid may be precipitated. This brings us to the chief value, in clinical medicine, of determining the composition of stones removed by operation. It enables more rational advice to be given as to the post-operative use of special diets, the administration of alkalis, etc., though it must be admitted that such treatment is generally of little value. In this connection the observations of McCarrison and of others are interesting. It would appear that in animals one of the factors responsible for the formation of urinary calculi (renal and vesical) is a deficiency of fat soluble vitamin.

Certain other substances may enter into the composition of stones. *Cystine* calculi are rare, and are due to an inborn error of metabolism (see *Cystinuria*), such stones contain a large proportion of the amino acid, and may be almost "pure", this is of practical importance, for it means that prolonged examinations for traces of cystine in calculi are unnecessary. *Xanthine* calculi are extremely rare, and may likewise be due to an inborn error of metabolism. *Calcium carbonate* stones are very rare in man, a number have been reported in herbivora.

### Determination of the Composition of Urinary Calculi

Most books on clinical diagnosis or physiological chemistry give a scheme for determining the composition of calculi. The following simplified procedure is quite satisfactory for clinical purposes — Wash the stone free from blood, mucus, preservative solution, etc., and dry it in the oven. Weigh it. Cut it in two, and note whether there is a foreign body (ligature, paraffin, etc.) which may have acted as nucleus. Reduce the stone (or half the stone if one half is to be kept for demonstration purposes) to a fine powder in a mortar. Perform the following tests with portions of the powder.

(1) **Heat on Platinum Foil** Organic matter (uric acid, cystine, xanthine, "albuminous matter," etc.) will burn away, inorganic matter (calcium, magnesium, phosphates, etc.) will remain<sup>1</sup>. All true calculi undergo more or less charring owing to the presence of varying amounts of organic matter. In a "pure" phosphate stone the only organic material may be the small amount of "albuminous matter". In this case carbonisation will be obvious only in the earlier stages of heating. Finally, the carbon will be all oxidised away, leaving a perfectly white residue. The larger the proportion of organic matter the greater the tendency to obtain finally a grey or black powder if moderate degrees of heat be used. Of course, all carbon can eventually be removed if heating is prolonged and a blow pipe be used.

If the powder burns away completely, or practically so, the stone will be composed mainly of urates (uric acid), or of cystine, or of xanthine. *Xanthine* is extremely rare, it does not give the murexide test. *Cystine* burns with a pale blue flame (It should be noted, however, that this may easily pass unnoticed). If cystine is suspected, a further portion of the original powder should be dissolved in a few drops of concentrated ammonia, cleared by centrifuging, allowed to evaporate spontaneously on a slide, and examined under the microscope. Typical hexagonal crystals clinch the diagnosis of cystine.

(2) **Heat some of the Original Powder with Dilute Hydrochloric Acid** (e.g. 10 per cent v/v). *Carbonates* will be detected at once owing to the evolution of carbon dioxide gas, human calculi occasionally contain mere traces, but, as a rule, none.

Uric acid, urates and albuminous matter will not dissolve in the dilute hydrochloric acid, calcium oxalate, cystine and phosphates will. Filter through a close paper (No 3 Whatman). Cool, add litmus and make the filtrate slightly alkaline with ammonia (phosphates and calcium oxalate, but not cystine, will be precipitated) and filter again (filtrate II). Shake the precipitate with a few cubic centimetres of water, and acidify with acetic acid. Phosphates will dissolve, but *calcium oxalate* will remain undissolved. Make filtrate II slightly acid with acetic acid, and, if necessary, add acetone a few drops at a time to make *cystine* crystallise out.

<sup>1</sup> Calcium oxalate will be decomposed leaving a residue of calcium carbonate (gentle heat) or calcium oxide (much heat).

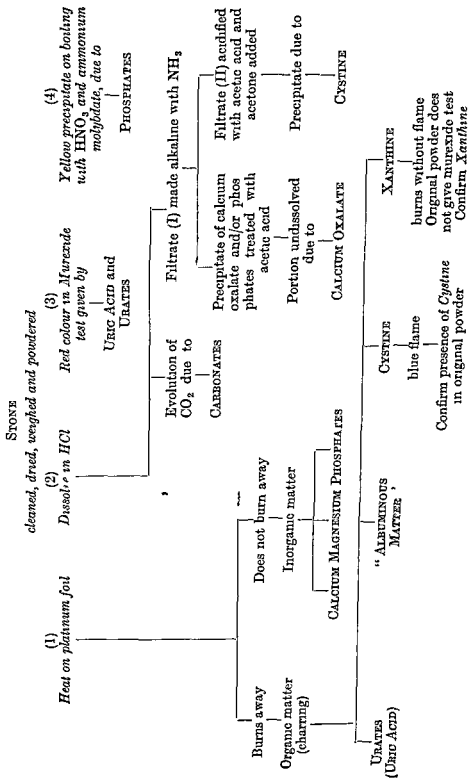
(cf Gaskell's Acetone Method for Cystinuria, p 53) Cystine will also have been identified in (1) Its presence at this stage in the precipitate on addition of acetic acid may be confirmed by microscopic examination for the hexagonal crystals The calcium oxalate precipitate may be amorphous or in the form of "envelope" crystals, depending on the rate of precipitation (Calcium oxalate and cystine are insoluble in acetic acid, but soluble in hydrochloric acid Phosphates are soluble in both acids Cystine is soluble, calcium oxalate is insoluble in ammonia )

(3) Heat some of the Original Powder in a Porcelain Dish on the Boiling-water Bath with a few Drops of Concentrated Nitric Acid Continue Heating until the Mixture is Absolutely Dry. A red colour is due to uric acid or urates (Murexide test) A yellow colour is of no significance

(4) To some of the Original Powder in a Test-tube add about 5 c c of 50 per cent v/v Nitric Acid and an Equal Volume of Ammonium Molybdate Reagent Boil A lemon yellow precipitate of ammonium phosphomolybdate indicates phosphates

The diagram on p 62 may make these directions more easy to follow

In a more detailed examination tests are also made for  $\text{NH}_4$ , Ca and Mg Ammonia may be present in combination either as ammonium urate or as ammonium magnesium phosphate, it is readily detected by adding 2 or 3 c c of sodium hydroxide solution to a large knife point of the powdered calculus and boiling, ammonia is revealed by its smell and by its action on moistened red litmus paper held at the mouth of the tube Examination for calcium and magnesium may be made by the ordinary methods of inorganic group analysis



## GALL-STONES AND MISCELLANEOUS CONCRETIONS

It is convenient to add here some notes on biliary calculi and various concretions

## Gall-stones

From a purely chemical point of view biliary calculi can be classified into three groups—cholesterol, pigment and calcium carbonate stones

**Cholesterol Stones** The great majority of gall stones are composed mainly of cholesterol, which often amounts to 90 per cent or more. They contain also varying traces of bile pigments (bilirubin and sometimes biliverdin), calcium, phosphates, proteins (chiefly mucus), and fatty material. The pigment is combined mainly, if not entirely, with calcium as calcium bilirubinate. These stones vary considerably in appearance and size, some are white, some are brown to black, they have a waxy feel.

**Pigment Stones** These are much less common, and are small and often black. They are composed mainly of calcium bilirubinate and oxidation compounds of bilirubin—biliverdin, bilifuscin and bilihumin. In some there are traces, in others no cholesterol at all. Protein and salts are present in small amounts, and there may be traces of iron and of copper.

**Calcium Carbonate Stones** These are very rare in man and consist mainly of calcium carbonate, together with calcium bilirubinate, there may be a trace of calcium phosphate. Unlike the previous two groups, they show up clearly on a direct X ray examination of the subject.

Bile salts are absent from gall stones, or occur in minute traces only.

The following is a simple scheme for testing for cholesterol, calcium, phosphates and bilirubin.

## Chemical Examination of a Gall-stone

Wash in water, dry and weigh. Note the appearance externally and on section, and reduce to powder in a mortar.

Heat a small portion of the powder on platinum foil. Most gall stones are very largely organic, catch fire and burn with a smoky flame and smell of burning animal fat, as is to be expected from their high content of cholesterol.

**Test for Cholesterol** Extract the rest of the original powder with ether in a boiling tube, using three or four portions each of about 5 c.c., dipping the tube each time into a beaker of water at 60° to 80° C., and filtering. Combine the filtrates and evaporate off the ether carefully (no flame). Crystallise the residue from the minimum of boiling 90 per cent alcohol. Mount the crystals under the microscope, and look for the typical notched plates of cholesterol containing one molecule of water of crystallisation (see Fig. 19 on p. 57).

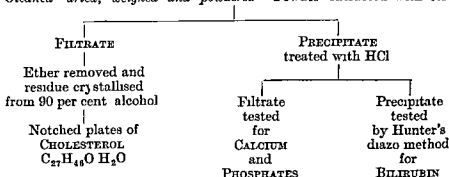
Should there be very little material extracted by ether and failure to produce crystals of cholesterol, a colorimetric test may be made as follows. Remove the 90 per cent alcohol completely by evaporation to dryness on a boiling water bath, extract the dry residue with 5 c c of chloroform, centrifuge to clear, decant and add 2 c c of A R acetic anhydride and 0.1 c c of concentrated sulphuric acid, a green colour is obtained if cholesterol is present (*cf* Estimation of Blood Cholesterol, p 351)

**Test for Calcium and Phosphates** Transfer the precipitate left on the filter after ether extraction, together with the paper, to the original boiling tube, add 2 to 10 c c of 10 per cent v/v HCl depending on the bulk of precipitate, warm and filter. Test half the filtrate for calcium by neutralising with ammonia, acidifying with acetic acid and adding a few drops of saturated ammonium oxalate, test the other half for phosphates by the molybdate test (see p 61)

**Test for Bilirubin** Wash the precipitate left after HCl extraction, together with any precipitate in the original boiling tube, with 10 c c of water into a centrifuge tube. Add a drop of saturated ammonium sulphate and 4 c c of 10 per cent  $\text{BaCl}_2$ . Centrifuge and remove the supernatant fluid with a test pipette. To the precipitate add 0.5 c c of diazo reagent (p 262) and mix. Add 2 c c of alcohol and 0.3 c c of 6 per cent  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , mix and centrifuge (*cf* Hunter's diazo test, p 19). A red colour, due to azobilirubin, shows that bilirubin is present.

#### GALL STONE

*Cleaned dried, weighed and powdered* Powder extracted with ether



#### Miscellaneous Concretions

**Urosteoliths and Fatty Concretions from the Urinary Tract.** These concretions have been reported on rare occasions. One consisted of paraffin derived from a paraffin bougie. The possibility of the formation of concretions from the local use of medicaments containing a fatty basis should be remembered. Others have been reported to contain neutral fat, free fatty acids, cholesterol, or mixtures of these substances.

"Pseudo-calculi" and "Pseudo-gravel." Patients occasionally

bring objects to their physician which they have discovered in the chamber, in order to find out whether they are passing "gravel." Occasionally a patient may deliberately attempt to deceive. Analysis of such fragments may be very difficult owing to the small amounts available. In general a careful microscopical examination combined with the tests outlined above under the "Determination of the Composition of Urinary Calculi" will enable an opinion to be given. The complete absence of any organic matter should arouse suspicion as to the nature of an alleged stone. The following experience illustrates the point. A patient brought minute "stones," some of which were black and some white, and claimed that she had passed them in the urine, and asked for advice as to treatment. On examination they were extremely hard, and were quite unaltered by heating on platinum foil. They contained no phosphate, oxalate, or urate. Carbonate and calcium were present, but no sulphates. There was not sufficient material to permit further analysis, but it was obvious that the fragments were not genuine urinary calculi.

**Enteroliths, and Concretions Passed per Rectum** *Gall stones* are occasionally passed in the faeces and it is mainly in order to establish whether or no a given "stone" passed per rectum is a gall stone that analysis of faecal concretions is required. The chemical identification of a true gall stone is generally easy (see above). Enteroliths (*cf* Morris) are usually hard rounded masses of food or lumps of faecal material encrusted with inorganic salts. The term "enterolith" is not meant to embrace lumps which are obviously small scybala (dried faeces). Sometimes bismuth or barium meals are followed, it may be several days later, by a passage of a ball of the particular salt used, probably coated with mucus and a little faecal matter. Tests for bismuth or barium make identification simple. Other concretions may be pieces of bone, masses of soaps or fats of high melting point, etc., etc.

**Salivary Calculi** On rare occasions a stone may form in one of the salivary ducts. Such stones consist of calcium phosphate or calcium carbonate or a mixture of these two substances. One examined by the writer consisted almost entirely of calcium phosphate, a small proportion of organic matter was present, but no calcium carbonate.

Tartar from teeth is said to be composed largely of calcium carbonate and phosphate, together with mucus, epithelial debris and organisms.

For descriptions of other types of concretions (pancreatic calculi, intestinal sand, corpora amylacea, etc.) the reader is referred to larger works, and particularly to Wells' *Chemical Pathology*.

### General Methods of Examination of Concretions

The clinical history, the source of the concretion, etc., will usually suggest the line of attack, but the following summary of methods may be useful —

(1) Naked eye appearance of the concretion as a whole and on section



(2) Action of organic solvents, *e g*, ether, alcohol, chloroform, etc Separation of ether-soluble matter from residue and examination of the two fractions.

(3) Examination as for urinary calculi, *viz*, heat on platinum foil, treatment with dilute hydrochloric acid, etc

(4) Special tests, *e g*, for soaps (removal of "fats" by ether, followed by acid hydrolysis of the residue, and a second extraction with ether), pigments, bismuth, barium, etc, etc.

### References

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## CHAPTER V

### TESTS OF RENAL EFFICIENCY

- Books.** Peters and Van Slyke's *Quantitative Clinical Chemistry*  
Ball and Evans' *Diseases of the Kidney*  
Fishberg's *Hypertension and Nephritis*  
*Recent Advances in Medicine Clinical, Laboratory, Therapeutic*, by  
Beaumont and Dodds  
Myers' *Practical Chemical Analysis of Blood*  
De Wesselow's *Chemistry of the Blood in Clinical Medicine*  
Thomson Walker's *Surgical Diseases and Injuries of the Genito  
Urinary Organs*  
'Modern Tests of Renal Function,' A. E. Belt Chapter x of  
Cabot's *Modern Urology*  
'Methods of Examination of the Functional Capacity of the Kidneys'  
Chapter xi of Kelly and Burnam's *Diseases of the Kidneys, Ureters and  
Bladder*

HAVING made a complete clinical examination of the patient, and having found protein in the urine together with an abnormal deposit, the medical officer may desire to extend his examination by applying tests of renal efficiency. Viewed in this light, i. e., as an extension of the clinical examination, renal efficiency tests are often of great value. Naturally in the majority of cases the chemical tests simply confirm the opinion already drawn from the clinical examination, but in so doing they give additional confidence. Occasionally the chemical tests are indispensable, or necessitate a revision of the conclusions drawn from the clinical examination.

There are one or two important points which should always be remembered in interpreting the results of renal efficiency tests—in fact, similar principles apply in testing the functional efficiency of any organ. In the first place, it is difficult or impossible to devise means of assessing the "reserve power" of the kidneys. No evidence of deficient function is obtained until a large part of the total renal tissue has been thrown out of gear. It is impossible to give an exact figure, but for practical purposes it is fair to state that until two thirds to three quarters of the renal tissue has been rendered functionless, the ordinary kidney efficiency tests yield "normal" results. In other words, if the tests are "normal," the kidneys may be healthy, or slightly damaged, or moderately deficient in function, but they cannot be seriously damaged. If the clinical examination, including careful urinary examination, is negative, then "normal" efficiency tests probably indicate healthy kidneys. But the results from chemical tests must always be assessed in the light of the clinical examination. If proteinuria accompanied by

granular or epithelial casts be found, the kidneys cannot be strictly healthy, however good be the efficiency tests

The converse of the above sometimes occurs. Is it possible, in the absence of both proteinuria and an abnormal deposit, for renal inadequacy to be present? In other words, does an unsatisfactory renal efficiency test have any significance when the urine is perfectly normal? Theoretically it is possible that the kidneys might be completely healed after a severe lesion, and so excrete urine of normal composition under ordinary conditions, and yet be so badly scarred that, when submitted to the extra strain of certain renal function tests, they would be shown to be deficient. In practice this condition must be extremely rare. The writer has never encountered a strictly normal urine when the kidneys were without doubt damaged, though, of course, the urine may be quite normal although one kidney has been removed surgically. It is said that at certain stages of chronic interstitial nephritis the urine may be free from protein, this is not my experience, though it must be admitted that sometimes there is so little protein that it may easily be missed, using the boiling and acetic acid test, unless the eye is trained readily to detect the slightest haze. In other words, the contradictory statements published on this point have probably arisen owing to errors of observation. In practice, therefore, one should always look carefully for fallacies in the chemical tests, if function tests are the sole evidence of renal inefficiency.

The second point of importance is that renal efficiency tests give no suggestion of the *cause* of deficient function. This may appear obvious, but is not uncommonly forgotten. Thus renal inadequacy may be due to nephritis, to stone, to tumours, etc., etc.

Thirdly, the possible influence of non renal factors on the results of tests of kidney function should always be borne in mind. Thus, if a substance is administered by mouth, the rate of its absorption from the intestine may be a factor in the rate of its excretion in the urine. Again, in the case of oral administration the liver may influence the amount reaching the kidneys. In the case of the ordinary excretory products, the kidneys may be functioning normally, but the amount excreted may be subnormal simply owing to a diminished production in the body. The possible influence of diet on the quantity of a substance excreted should never be forgotten. Moreover, patients exhibit variations of appetite and peculiarities of taste at least as much as healthy individuals. Be not misled, therefore, by the statement that any particular patient was on a "full hospital diet" at the time of the test.

Lastly, it is necessary repeatedly to make observations if the progress of a case is to be judged from chemical tests. An isolated test gives information as to the state of the kidneys only on the particular day on which that test was made.

#### Objects of Kidney Function Tests

- (1) Differential diagnosis from non renal diseases
- (2) In unilateral disease, diagnosis of the side affected

(3) Estimate of degree of damage to each kidney separately, or estimate of damage to one kidney and "proof" that the other is functionally efficient

(4) Estimate of total damage estimate of risk of uræmia

(5) Repeated estimations of kidney function (unilateral or combined) at intervals as an aid to prognosis, or,

(6) As an aid to treatment

### Conditions of Testing

Tests may involve the examination of the "combined urine" from the bladder, with or without catheterisation, or of the segregated urines obtained by ureteric catheterisation, or of the blood. Lastly, examinations of blood and urine may be combined in order to ascertain the state of affairs on both sides of the kidneys.

The substance estimated may be an ordinary constituent of the blood and of the urine, or it may be an artificial product administered by mouth or parenterally for the purpose of the test, *e.g.*, dye tests. In practice estimations on the urine have very largely been given up, owing to the difficulty of obtaining a complete collection of the twenty four hours' urine. Finally provocative tests may be employed. In such tests, relatively large doses of substances are administered in order to force the kidney to work hard. In this way some measure of the reserve power of the renal tissue is obtained, the kidney is working at a disadvantage, and defects in function may be rendered obvious which would escape notice using other tests. An example of a provocative method is the urea concentration test.

### The Functions of the Kidneys

The main functions of the kidneys are —

(1) EXCRETION of

✓(a) Waste products of metabolism

(b) Poisons, chemical and bacterial (these may or may not have been rendered innocuous by the liver before reaching the kidneys) ✓

✓(c) Other foreign substances

✓(2) Regulation of the OSMOTIC PRESSURE of the blood, chiefly by control of salts excreted, especially chlorides

✓(3) Regulation of the HYDROGEN ION CONCENTRATION of the blood

✓(4) CONSERVATION OF THE BASES—particularly sodium—of the blood and body fluids. The chief mechanism is the conversion of urea into ammonium carbonate in the kidneys, a double exchange then takes place, the ammonium salt is excreted in the urine, and the sodium is conserved as bicarbonate in the blood (*cf* p 184)

(5) KIDNEY SYNTHESIS, for example, the conjugation of benzoic acid with glycine to yield hippuric acid (benzoyl glycine)

## TESTS EMPLOYED

As a preliminary, search will have been made for abnormal constituents in the urine, particularly protein, cells and casts (Chapters III and IV). If the urine is *absolutely* free from protein it is a waste of time to perform renal function tests. An enormous number of methods have been devised for testing one or more of the functions listed above. It is impossible in a book of this size to give particulars of all these. Some of the methods most commonly employed in this country will be discussed in detail. Notes will be included on certain others. Lastly, references will be given to further tests of interest.

Nowadays the urea tests are more widely used than any of the others, and it is probably fair to state that they can be relied on exclusively in routine work. The value of other methods in special circumstances (*e.g.*, the dye tests in the hands of the surgeon himself) will be noted as the tests are described.

## THE UREA CONCENTRATION TEST (MACLEAN AND DE WESSELOW)

**Principle.** The object of this test is to force the kidneys to *concentrate* urea by flooding the blood with urea, and by withholding fluids for eight to twelve hours beforehand.

**Technique****(a) Performance of Test in Ward, etc.**

Nothing to drink overnight, *e.g.*, nothing after 10 p.m. Next morning at —

5.58 a.m.	Empty bladder completely Label specimen "0"
6 a.m.	Drink the dose of urea.
7 a.m.	Empty the bladder again Label specimen "1"
8 a.m.	Pass urine again Specimen "2"
9 a.m.	Pass urine once more Specimen "3"

Place each specimen in a separate bottle and label each.

The whole of the urine passed in specimens 1, 2 and 3 should be sent. A sample of specimen "0" will suffice. Breakfast may be taken before or during the test if desired, so long as it is a "dry" breakfast. No tea, coffee, milk, etc., is allowed.

The test may be started at 6.30 a.m. or 7 a.m., or at any other convenient time in the early morning, so long as complete samples of urine are obtained at hourly intervals as in the above example, and so long as all fluids are withheld previously for eight to twelve hours.

Urea prescriptions for an adult —

R Urea ✓ 3 jvss  
 Tinct aurant ✓ m xv  
 Aquam ✓ ad 3 iijss  
 M Ft Haust ✓

or,

R Urea 15 gm  
 Tincture of orange 1 c c  
 Water to 100 c c

Dose of urea for children The following has been found satisfactory in practice —

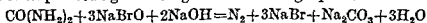
Age in years	c c of above prescription	Grammes of urea
12 and over	100	15
8 to 12	80	12
5 to 8	70	10 (approx )
3 to 5	50	7½
1 to 3	40	6
Under 1	25	4 (approx )

(b) *Laboratory Determinations* Measure the volume of urine in cubic centimetres and estimate the percentage of urea in each of specimens 1 2 and 3 The volumes are measured in order to detect excessive diuresis The hypobromite method for the estimation of urea is sufficiently accurate for clinical purposes in that only relatively gross variations in urea concentration have any significance ✓

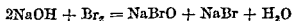
### Technique of Estimation of Urea in Urine

#### (Hypobromite method)

**Principle** Nitrogen is liberated from the urea in urine by the action of an excess of alkaline sodium hypobromite and is collected over water, the volume is measured at atmospheric pressure, the reaction proceeding according to the following equation —



**Reagents** A freshly prepared solution of alkaline sodium hypobromite is made by mixing 1 part of bromine (2.5 c c ampoule) with 10 parts of 40 per cent sodium hydroxide solution (25 c c), i.e., an excess of caustic soda



The reagent must be freshly prepared each day

**Apparatus** (see Fig 20 on p 72) The apparatus consists of a 100-c c burette connected at the lower end by rubber tubing with

a levelling tube, and at the upper end with a small bottle. This is closed by an indiarubber bung with two holes. Through these pass two short pieces of glass tubing. One of these carries the rubber tubing making connection with the upper end of the burette as already mentioned, the other is closed by a short length of rubber tubing and a screw clip. The bottle contains a small test-tube which will just lie obliquely across the bottle without spilling its contents.

**Method** Measure approximately 25 c.c. of the freshly prepared sodium hypobromite cooled to room temperature, and transfer to

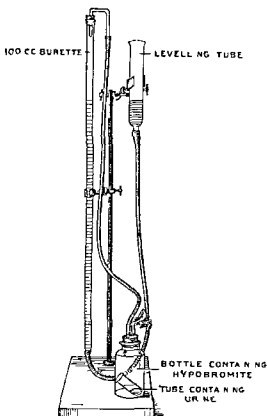


FIG 20 Urinary urea apparatus (hypobromite method)

the bottle. Measure accurately 5 c.c. of the urine with a pipette, and deliver it into the small test tube. Carefully lower this into the bottle, taking care to avoid spilling urine into the hypobromite. Place the rubber bung securely in place, and, with the screw clip open, adjust the levelling tube until the water in the burette is at zero. Close the clip. The air inside the system is now at atmospheric pressure. Lower the levelling tube. The water in the burette will drop a few divisions and should then remain stationary. If, however, it continues to fall an air leak is present which must be eradicated before the experiment can be continued.

Having made certain that the system is air tight, mix the urine

with the hypobromite by tilting the bottle, taking care to prevent froth entering the mouths of the glass tubing passing through the indiarubber bung, mix thoroughly and allow the mixture to stand for two or three minutes to cool approximately to room temperature. Adjust the levelling tube until the levels of the water in it and in the burette are the same. The gas in the burette will then be at atmospheric pressure. Read the burette.

**Calculation.** The urea percentage is obtained by dividing the volume of nitrogen evolved by 20; 1 c.c. of nitrogen corresponds to 0.05 per cent. of urea; this factor, of course, only holds if 5 c.c. of urine are used per test.

In other words, under the conditions of the experiment (room temperature, etc.), 1 c.c. of nitrogen is equivalent to 0.0025 gm. of urea. Therefore, if 1 c.c. of urine be used per test, each cubic centimetre of nitrogen corresponds to  $0.0025 \times 100$  grammes of urea per 100 c.c. of urine; and with 5 c.c. of urine, to  $0.0025 \times 20 = 0.05$  per cent.

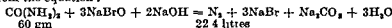
When 4 c.c. of urine are used per test, as by MacLean and de Wesselow in their original publication, each cubic centimetre of nitrogen corresponds to  $0.0025 \times \frac{100}{4}$  or 0.0625 per cent. of urea.

Five c.c. pipettes are more often available than 4 c.c.—hence the adoption of 5 c.c. of urine per test in the above description.

**Note on Factor.** This was determined by MacLean and de Wesselow in the following way. The urea percentage in each of a series of urines was determined by the accurate urease method. The nitrogen evolved from 4 c.c. of each of the same urines was then measured at room temperature and without correction for barometric pressure. The relation of nitrogen evolved to the urea percentage in each urine was calculated, and the results averaged. It can readily be shown by calculation that this factor may safely be employed even in hot climates, the temperature error being too small to interfere with the value of the results in clinical work.

The above technique is a simplification of the standard procedure, in which the burette is immersed in a large cylinder of water so that the temperature can be measured (see, for example, Cole's *Practical Physiological Chemistry*). The chief difficulty, as a rule, in the standard method is the calculation, which is therefore appended—

From the equation:



60 gm

22.4 litres

60 gm. of urea theoretically would yield 22.400 c.c. of nitrogen, or 1 gm. of urea would yield 373 c.c. of nitrogen at S.T.P. In practice it is found that a solution of 1 gm. of urea in water gives off only 357 c.c., or about 96 per cent. of the theoretical amount. In urine, too, the urea yields about 96 per cent. of the nitrogen demanded by theory, but the alkaline hypobromite liberates nitrogen also from ammonium salts, amino acids, uric acid and creatinine (cf. Stehle, R. L., *J. Biol. Chem.*, 1921, 47, 13), and thus extra nitrogen just about balances the deficiency in nitrogen from urea. In practice, therefore, the above equation may be made the basis of calculation. It is of interest to note, too, that MacLean and de Wesselow's factor (see above), viz., 1 gm. of urea is equivalent to 400 c.c. of nitrogen at room temperature, when reduced to S.T.P. again approximates to 1 gm. of urea  $\equiv$  373 c.c. of nitrogen.

Let the temperature at which the nitrogen is collected be  $t^\circ \text{C}$ ,  $p$  being the barometric pressure,  $a$  the aqueous vapour pressure at the observed temperature, and  $v$  the volume of nitrogen evolved from 5 c.c. of urine.

Then the corrected volume of nitrogen  $V$

$$= v \times \frac{273}{273 + t} \times \frac{p - a}{760}$$



Since 373 c c of  $N_2$  are evolved from 1 gm of urea,

$$\begin{array}{ccccccc} 1 & & " & & " & & \frac{1}{373} & & " \\ & & & & & & \frac{V}{373} & & " \end{array}$$

Therefore 5 c c of urine contain  $\frac{V}{373}$  gm of urea

$$100 \quad , \quad " \quad \frac{V \times 20}{373} \text{ gm of urea}$$

Or substituting for V and calculating in one operation —

$$\begin{aligned} \text{Urea per cent} &= v \times \frac{273}{(273 + t)} \times \frac{(p - a)}{760} \times \frac{20}{373} \\ &= \frac{v \times (p - a)}{(273 + t)} \times \frac{273 \times 20}{760 \times 373} \\ &= \frac{v \times (p - a)}{(273 + t)} \times 0.0193 \end{aligned}$$

**Interpretation of Results** (a) **Volume of Urine.** If the concentration of urea is subnormal, the first thing to exclude as a cause thereof is excessive diuresis. The volume of urine should not exceed 120 c c (4 oz) in the first hour, or 100 c c ( $3\frac{1}{2}$  oz) in the second and each subsequent hour. If the volumes do exceed these amounts, a low urea concentration is *not necessarily* indicative of deficient renal function, and it is advisable to repeat the test. Excessive diuresis is most commonly due to imperfect preparation for the test. Occasionally, in spite of withholding fluids for eight to twelve hours previously, there may be excessive diuresis owing to release of water previously retained in the tissues. It is merely a matter of convenience whether the test be repeated on the same or on another day, there being no objection to giving a second dose of urea two or three hours after the first. If the urea concentration be good in spite of excessive diuresis, there is obviously no need to repeat the test.

(b) **Concentration of Urea** The information required is the *maximum concentration* of urea. It does not matter whether this occurs in the first, second or third hour. Usually the maximum is found in the second or third, but occasionally in the first hour. It is, therefore, advisable to determine the percentage of urea in each of the three hours but if time is pressing the second or third hour sample may be analysed first, and if satisfactory further analysis may be omitted.

Normally the maximum concentration exceeds 2.5 per cent, frequently being greater than 3 per cent. Figures between 2.5 and 2 per cent may be regarded as within the boundary zone. Their significance should be carefully assessed in the light of all the evidence available, and if any doubt remains as to the efficiency of the kidneys further tests should be performed, e.g., an estimation of the blood urea. Figures below 2 per cent generally indicate renal inadequacy, always assuming that the test has been properly executed.

**Value of the Test** On account of its simplicity the test has been used extensively in all branches of urinary disease. It is very

useful in clinical work, and undoubtedly will reveal the presence of a serious degree of renal inefficiency in the vast majority of cases. There are, however, a few groups of cases in which it is apt to be misleading at first sight. Thus in the purely hydræmic type of nephritis with chloride retention, but no nitrogenous retention, it gives normal results, and yet the patient may be at death's door. Again, in some cases of enlarged prostate the urea concentration test has been normal before operation, and yet the patient has died from septicæmia or toxæmia twenty four or forty eight hours after prostatectomy. The test is a measure of the efficiency of nitrogenous excretion, and of that only. We have no right, therefore, to expect it to give warning of septic complications, etc.

The urea concentration test is a provocative test. That is to say, by flooding the blood with urea an attempt is made to provoke the kidneys to excrete urea at higher concentrations than on an ordinary diet. It is possible that in the case of a damaged kidney such provocation makes a call upon some of its "reserve." Possibly for this reason the urea concentration test is more sensitive than many other tests, such as the blood urea test. But even the urea concentration test is a comparatively gross test. Thus in one case the writer examined it was perfectly normal, although one of the kidneys had been removed following a street accident. It would appear, therefore, that half the renal tissue may be functionless, and yet this test will yield "normal" results.

### BLOOD UREA

The concentration of urea in the blood is determined to see whether there is a retention of urea.

**Collection of Blood** (*cf* Chapter XVII). Venepuncture is necessary to obtain enough blood for the hypobromite method (5 c c blood) and for the "urease titration" method (3 c c), but capillary blood secured by skin puncture may be used for the "urease Nesslerisation" method (0.2 c c). Fluoride must *not* be used for preserving the blood when urease methods are to be employed, because it is an enzyme poison and may cause low results.

It does not matter whether whole blood, oxalated plasma or serum be used, because urea is approximately evenly distributed between corpuscles and plasma. Urea diffuses very readily, and the urea content of the blood and of the various body fluids are practically uniform. This point is of practical importance in that cerebrospinal fluid or œdema fluid may have already been obtained and be available for analysis. It has even been suggested that the saliva be utilised for the estimation of urea, which is calculated from the combined urea and ammonia nitrogen (Hench and Aldrich).

### Methods of Estimation

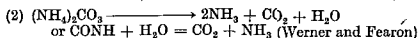
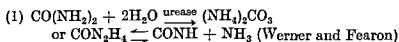
For a review of the available techniques, see Peters and Van Slyke's *Quantitative Clinical Chemistry*, vol. II.

## I. Hypobromite Method

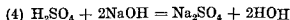
The proteins in 5 c c of blood are precipitated by trichloroacetic acid. An aliquot part of the protein free filtrate is treated with sodium hypobromite in a special apparatus. The nitrogen liberated is measured and the percentage of urea calculated. Gross departures from the normal are readily detected, but the method is not so accurate as the urease methods, which are, therefore, recommended in preference.

## II Urease-titration Method

**Principle** Blood is incubated with urease in a solution of acid potassium phosphate which preserves the optimum reaction for the enzyme. The urease converts the urea into ammonium carbonate. An excess of weak base (potassium carbonate) is added, and the ammonia liberated is aspirated over into an excess of standard acid. The excess of acid is determined by titration with standard alkali. The rest of the acid has been neutralised by the ammonia formed from the urea. *The amount of preformed ammonia in blood is so minute (see Table on p. 333) that it can be disregarded*, in other words there is no necessity to estimate (1) the preformed ammonia, and (2) the ammonia preformed plus that formed from urea as in the case of urine.



(Dissociation hastened by presence of  $\text{K}_2\text{CO}_3$ , removal of  $\text{NH}_3$  by aeration and fixation of  $\text{CO}_2$  by  $\text{K}_2\text{CO}_3$  ( $\text{K}_2\text{CO}_3 + \text{H}_2\text{O} + \text{CO}_2 = 2\text{KHCO}_3$ ))



**Apparatus** The apparatus consists of three vessels connected in series as shown in Fig. 21. The guard bottle A contains sulphuric acid (10 to 25 per cent v/v) to trap any ammonia drawn in from the laboratory atmosphere. The boiling tube B contains the blood, the urease, and the acid phosphate. The boiling tube C contains the standard acid. The suction pump P draws the air through the contents of the tubes, and carries  $\text{NH}_3$  from B into C.

**Method** In tube C place 25 c c of N/100 sulphuric acid, 4 or 5 drops of indicator,<sup>1</sup> and 2 or 3 drops of caprylic alcohol. In tube B place 5 c c of 0.6 per cent acid potassium phosphate, 3 c c of

<sup>1</sup> Indicator. Prepared by adding 1 c c of saturated aqueous methylene blue to 50 c c of saturated methyl red in 50 per cent alcohol.

blood, 0.3 gm of soya bean meal, and 10 to 12 drops of caprylic alcohol (the last to prevent frothing). Connect up the apparatus as shown in Fig 21. See that the inlet tubes are properly arranged so that the contents of one boiling-tube are not sucked over into the next. Place tube B in a water bath at  $48^{\circ}\text{C}$ . The temperature may be

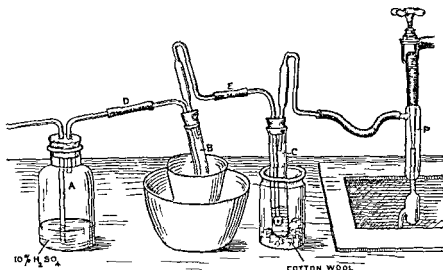


FIG 21 Blood urea apparatus for urease titration method

maintained for a longer period if the inner water bath at  $48^{\circ}\text{C}$  is placed in an outer water bath at  $60^{\circ}\text{C}$  as shown in the figure, enamel basins serve well as water baths. (Alternatively, clips may be applied at D and E, and the tube B with its clipped rubber connections may be placed in an incubator.) Incubate for half an hour, shaking tube B, and aspirating air through by turning on the pump for a few seconds, about every seven minutes (four times in the half hour), so that its contents are thoroughly mixed.

At the end of the period of incubation, the contents of tube B are made alkaline as follows. Remove the water baths from under tube B, and disconnect this tube, blowing through A at the moment the tube is drawn down, so that the contents of B are not sucked up towards A, and so that no drop is left behind to fall on the bench if the boiling tube is taken away. To B add rapidly 3 gm of solid anhydrous potassium carbonate, and 4 cc of saturated aqueous potassium carbonate. Quickly replace the tube, and put the water baths back in their place. Then aspirate air through the whole system by turning on the suction pump slowly for five minutes, and then more rapidly so that a good stream of air passes. Aerate altogether for forty five to sixty minutes.

Then disconnect C, blowing down A so that no fluid is sucked back from C towards B. Undo the rubber connections between the pump and C, and between C and B. Holding the bulb just above the level of the acid, wash the inside and outside of the glass tube which had been in the acid, with a jet of distilled water, down

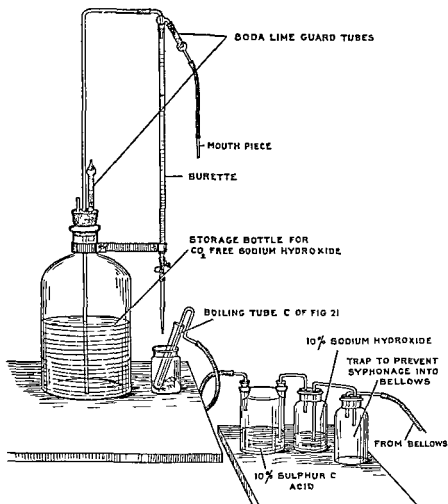


FIG 22 Titration in  $\text{CO}_2$  free air Storage bottle and burette for  $\text{CO}_2$  free  $\text{NaOH}$

into the contents of C Titrate the excess of acid with  $\text{N}/100$   $\text{NaOH}$  in  $\text{CO}_2$  free air, using the apparatus shown in Fig 22 above The end point is shown by a change of colour from purple to green

**Blank** The soya bean meal contains a trace of ammonium salts A blank is performed to check this and the other reagents, by carrying out the above procedure, except that 3 c.c. of distilled water are substituted for 3 c.c. of blood The blank calculated as urea per 100 c.c. should lie between 2 and 5 mgm, i.e. a back titration of 24.8 to 24.5 c.c. of  $\text{N}/100$   $\text{NaOH}$  or a caustic soda deficiency of 0.2 to 0.5 c.c.  $\text{N}/100$  It is only necessary to repeat the blank when fresh reagents are employed

**Calculation** Using 3 c.c. of blood and exactly  $\text{N}/100$  sulphuric acid and sodium hydroxide —

Blood urea = (c.c. caustic soda deficiency  $\times 10$ ) less the blank value in terms of urea

Thus, if the back titration with  $\text{N}/100$   $\text{NaOH}$  is 21.9 c.c., the "soda deficiency" =  $25 - 21.9 = 3.1$  c.c. Suppose the blank

corresponds to 3 mgm of urea per 100 c c, then the blood urea =  $(31 \times 10) - 3 = 28$  mgm per 100 c c

### Example of Calculation from First Principles —

3 c c of whole blood used

25 c c of 0.01065 N  $\text{H}_2\text{SO}_4$  used to catch the ammonia

Back titration was 22.4 c c of 0.00992 N caustic soda

Blank corresponds to 2 mgm of urea per 100 c c

1,000 c c of N/1  $\text{H}_2\text{SO}_4$  combine with 17 gm of  $\text{NH}_3$  or 14 gm of ammonia N

1 c c of N/1  $\text{H}_2\text{SO}_4$  is equivalent to 14 mgm of nitrogen

1 c c of N/100  $\text{H}_2\text{SO}_4$  is equivalent to 0.14 mgm of nitrogen

Convert acid and alkali into N/100 thus —

$$25 \times 0.01065 = x \times 0.01, \text{ wherefore } x = 26.625$$

$$\text{and } 22.4 \times 0.00992 = y \times 0.01, \text{ wherefore } y = 22.221.$$

Therefore, the acid which has been neutralised by the ammonia formed from the urea is  $26.625 - 22.221$  c c, or 4.404 c c N/100

Since 1 c c of N/100 acid corresponds to 0.14 mgm of ammonia N

4.404 c c of N/100 acid corresponds to  $4.404 \times 0.14$  mgm of N,

or, 3 c c of blood yielded  $4.404 \times 0.14$  mgm of ammonia N

$$\begin{array}{ccccccc} & & & 4.404 \times 0.14 & & & \\ & 1 & & & 3 & & \\ & & & & & & \end{array}$$

$$\begin{array}{ccccccc} & & & 4.404 \times 0.14 \times 100 & & & \\ & 100 & & & 3 & & \\ & & & & & & \end{array}$$

Each 60 gm of urea yield 28 gm of ammonia N

$$100 \text{ c c of blood yield } \frac{4.404 \times 0.14 \times 100 \times 60}{3 \times 28} \text{ mgm of urea,}$$

i.e., 100 c c of blood contain  $4.404 \times 10$  mgm urea = 44 mgm

But from this we must subtract 2 mgm for the blank value<sup>1</sup>

Therefore, blood urea = 42 mgm per 100 c c

**Notes Indicator** Methyl red is red in acid, yellow in alkaline solution. The change from red to yellow, particularly in artificial light, is not sharp. The addition of methylene blue makes the end point more obvious. The methylene blue does not alter in colour. Red + blue = purple, changes to yellow + blue = green.

**Urease** The soya bean is a convenient source of urease. The meal is cheaper than the prepared tablets of urease and generally keeps well in Great Britain. The soya beans are ground up into a meal, which may be purchased, e.g., from Messrs Baird & Tatlock, Ltd. The meal is passed through a forty mesh sieve, and the coarse particles rejected.

N/100 sulphuric acid keeps well in paraffin lined or hard glass bottles. It must be checked against the caustic soda every two or three months.

<sup>1</sup> This method of allowing for the blank value in the calculation obviously only holds when 3 c c of blood are used.

$\text{CO}_2$  free N/100 caustic soda is prepared and standardised against pure acid potassium phthalate as described by Cole (*Practical Physiological Chemistry*)—see Appendix It keeps well in paraffin-lined bottles The combined storage bottle and burette as shown on p 78 is very convenient The NaOH should be restandardised every two or three months

### III Urease-Nesslerisation Method (Archer and Robb)

**Principle** Blood is incubated with urease to convert the urea into ammonium carbonate The proteins are precipitated by tungstic acid, and an aliquot part of the supernatant fluid is treated with Nessler's reagent Standard solutions of an ammonium salt are also treated with Nessler's reagent, and standards and unknown are compared colorimetrically

**Method** In a non tapered centrifuge tube place 2 c c of distilled water and 0.2 c c of blood, which is measured with an accurate blood pipette calibrated "to contain", and is delivered beneath the 2 c c of water The pipette is then raised and washed out with the water two or three times Add 0.2 c c of the urease suspension<sup>1</sup> (prepared by grinding up one urease tablet in 5 c c of 30 per cent alcohol) and shake well to mix

Place the centrifuge tube in a water bath at 55° C for fifteen minutes (or in a beaker of water which has been heated to 60° C)

Remove the tube and add 0.3 c c of 10 per cent sodium tungstate, 0.3 c c of two thirds normal sulphuric acid and 5 c c of distilled water Shake well to mix, stand a few minutes till the protein precipitate flocculates, and centrifuge till the supernatant fluid is quite clear Pipette off 5 c c of the supernatant fluid into a clean test tube, and add 5 c c of water and 2 c c of Nessler's reagent

The standards are prepared by mixing —

(A) One cubic centimetre of ammonium sulphate\* standard solution, 9 c c of water and 2 c c of Nessler's reagent, and

(B) Two cubic centimetres of ammonium sulphate standard solution, 8 c c of water and 2 c c of Nessler's reagent

A solution of ammonium sulphate is kept in stock which has been prepared by dissolving 2.2 gm of the pure dry salt in water and making up to 1,000 c c

The ammonium sulphate standard solution is prepared by diluting this stock solution 1 in 20 by taking 5 c c and making up to 100 c c with water For preparation of Nessler's reagent, see Appendix

Compare the colours of the "unknown" and of the standards with the unaided eye, and select the standard approximating most nearly to the unknown Place the unknown in the left hand cup of the colorimeter and set the reading at 40 mm Place the selected standard in the right hand cup, which move up and down in the usual way till the colours match Take a series of readings, of which let the average be S

<sup>1</sup> Alternatively a large knife point (0.1 gm) of soya bean meal (remove coarse particles by a 40 mesh sieve) may be used plus 0.2 c c of distilled water

**Calculation** The reading of the unknown is 40, that of the standard is S. Standard A corresponds to 40 mgm of urea per 100 c c. Standard B corresponds to 80 mgm of urea per 100 c c. Therefore,

$$\text{Blood urea} = \frac{S}{40} \times 40 = S \text{ mgm per 100 c c (A),}$$

$$\text{or,} \quad \frac{S}{40} \times 80 = 2S \quad \text{,,} \quad \text{,,} \quad \text{(B)}$$

**Calculation from First Principles** Using standard A (1 c c of 0.011 per cent ammonium sulphate) —



132 gm of ammonium sulphate contain 28 gm of nitrogen

60 gm of urea contain 28 gm of nitrogen

132 gm of ammonium sulphate correspond to 60 gm of urea

1 gm of ammonium sulphate corresponds to  $\frac{60}{132}$  gm of urea

0.11 mgm of ammonium sulphate (or 1 c c of 0.011 per cent) corresponds to  $\frac{60}{132} \times 0.11 = 0.05$  mgm of urea

1 c c of 0.011 per cent ammonium sulphate contains nitrogen corresponding to 0.05 mgm of urea

Let  $x$  be the quantity (in milligrammes) of urea in the 5 c c of supernatant fluid used,  $S$  be the reading of the standard, and  $U$  be the reading of the unknown

Then  $S \times 0.05 = U \times x$ ,

$$x = \frac{S}{U} \times 0.05 \text{ mgm}$$

5 c c of supernatant fluid are derived from  $\frac{5}{8} \times 0.2$  c c of blood

$\frac{1}{8}$  c c of blood contains  $\frac{S}{U} \times 0.05$  mgm of urea

$$\begin{aligned} \therefore 100 \text{ c c} \quad \text{,,} \quad \text{,,} \quad & \frac{S}{U} \times 0.05 \times 800 \text{ mgm of urea} \\ & = \frac{S}{U} \times 40 \text{ mgm of urea} \end{aligned}$$

Wherefore, if the unknown is set at 40 mm,  $U = 40$  and 100 c c of blood will contain  $S$  mgm of urea. Also if the standard and unknown match exactly, the blood must contain 40 mgm of urea per 100 c c, i.e., standard A corresponds to 40, and standard B to 80 mgm of urea per 100 c c, if, and only if, the measurements given above are adhered to.

**Blank** Perform a blank test on the reagents with each new batch of urease tablets, or of soya bean meal, by proceeding exactly as above, except that 0.2 c c of water is used instead of blood.



The tablets as a rule give no perceptible colour, *i.e.*, blank is *nil*. The soya bean meal may give a very slight colour, but from the measurements given previously it will be seen that the large knife-point (or 0.1 gm.) of soya bean meal should introduce an error corresponding to +1 to +2 mgm. of urea per 100 c.c., which may be neglected for routine clinical work.

**Notes** The protein precipitate must be removed by centrifuging and not by filtration, because filter papers, unless specially washed, contain ammonium salts, and give a positive colour reaction with Nessler's reagent.

All water used in the test and for making solutions must, of course, be free from ammonia. Freshly distilled water is usually safe, but each supply should be tested with Nessler's reagent.

The chief difficulty of the method lies in the colour matching, brown being a poor colour for colorimetric work. Daylight, or artificial light with a daylight glass, may be used. Results obtained by this method (0.2 c.c. of blood) have been compared with those by the urease titration method (3 c.c. of blood), the agreement has been very close in a series of over 100 bloods.

When the urea content is high, the blood should be diluted to comply with the fundamental condition for colorimetry, *viz.*, that the colour of the unknown shall approximate to the colour of the standard (see Chapter I).

If the blood urea exceeds 200 mgm. per 100 c.c., the supernatant fluid may not clear on centrifuging, or the final addition of Nessler's reagent may cause an opalescence, the probable cause of this is the neutralisation of an appreciable fraction of the  $\frac{3}{8}N$   $H_2SO_4$  by the ammonium carbonate formed from the urea, so that the proteins are not completely precipitated (for each 100 mgm. of urea per 100 c.c., 0.01 c.c. of  $\frac{3}{8}N$   $H_2SO_4$  are thus neutralised). In such cases, and whenever it is anticipated that the blood urea will be high, use 0.1 c.c. of blood plus 0.1 c.c. of water.

Another possible cause of cloudiness on Nesslerisation is acetone which has not been removed completely during the drying of pipettes ("mercury acetone" precipitate).

The urease suspension in 30 per cent. alcohol usually keeps for at least a week at room temperature, but in hot weather loss of activity has been noted in a shorter period. If water is used for making the suspension, loss of potency occurs in two or three days. Tablets, either of "B D H urease" (one tablet  $\equiv$  50 mgm. urea) or of "urease Dunning" are satisfactory.

### Interpretation of Blood Urea

The concentration of urea in the blood is very little influenced by ordinary mixed meals. It does not matter, therefore, at what time of the day the blood is obtained. The blood urea, however, though slowly, is sometimes extensively affected by great reduction of protein in the diet. For this reason the blood urea may be misleading in cases of kidney disease. Cases of uræmia have been reported in which the blood urea was reduced from very high values

to within normal limits solely as a result of the restriction of proteins. The writer has occasionally experienced the same difficulty in cases of enlarged prostate with severe anorexia. For this reason the estimation of blood urea at a known interval after a standard dose of urea has been advocated. A convenient method is to combine the urea concentration test with the estimation of urea in the blood—see under Urea Concentration Factor (p 98). Urea administered by the mouth is rapidly absorbed. At the end of an hour, after 15 gm., the blood urea is approximately doubled in a normal person. Archer and Robb suggest that a blood urea curve would be of value.

The chief difficulty in interpreting the meaning of an increase in the urea content of the blood lies in the exclusion of non renal causes of increase. Thus severe diarrhoea, gastro enteritis, etc., and, in fact, any cause of severe dehydration, tend to raise the blood urea. Again, a failing circulation has a similar effect, in this case owing to the diminution of urinary secretion as a result of the fall in blood pressure. In addition to cardiac cases proper, the circulation is deficient in the terminal stages of many diseases. For these reasons the interpretation of urea in blood taken shortly before or after death is often difficult. Another cause of slightly raised values is excessive protein destruction, *eg*, in diabetics submitted to severe undernutrition. The rise of blood urea in these non renal cases is usually only moderate, of the order of 50 to 100 mgm per 100 c c of blood. As MacLean has emphasised, they may easily be distinguished by estimating the concentration of urea in the urine, which will be found to be high, often exceeding 4 per cent. Another cause of a raised blood urea is intestinal obstruction (see Chapter IX).

Normally, the figures for the concentration of urea in the blood are as follows —

	mgm per 100 c c
Adult, on a "full" diet	15-40
Under ten years, on a "full" diet ( <i>cf</i> Table p 95)	15-50
Over sixty years, on a "full" diet	15-50
Two and a half hours after 15 gm of urea	40-80

The metabolism of children is more vigorous than that of adults, and this may be the cause of the higher figures. In old age the higher blood urea is probably due to the kidneys being less efficient.

### Value of the Test

An estimation of urea in the blood, like the urea concentration test, is a measure of the efficiency of nitrogenous excretion and of that only. The remarks made in this connection in discussing the value of the urea concentration test apply with equal force to the blood urea.

It will be seen from the above that owing to the difficulties of

interpretation the value of an estimation of the blood urea is limited. None the less, it is the simplest of the "retention" tests, and its extensive employment has led to wide recognition of its limitations. In proved kidney cases it is very valuable, both in watching the progress of a case and as a guide to treatment. In the diagnosis of uræmia it is very useful, and there is generally little doubt as to the meaning of really high figures, *e.g.*, over 100 mgm. In all doubtful cases its value is greatly enhanced if combined with an estimation of the concentration of urea in the urine, either as part of the urea concentration test or even in isolated specimens of urine. In fact, when used as an aid to diagnosis it is a good rule always to perform urinary estimations if the blood urea lies between 50 and 100 mgm. The latest development of this principle is seen in the next test.

## THE BLOOD UREA CLEARANCE TEST

(Van Slyke, *et al*.)

By this method are measured the cubic centimetres of blood which would be completely cleared of urea during passage through the kidneys, if such a clearance were actually complete. Really, of course, the kidneys never remove completely the urea from the blood, there is a lower urea concentration in the renal vein than in the renal artery, but urea is never absent from the blood in the renal vein. None the less the theoretical conception is a useful measure of renal efficiency, the larger the volume of blood which could, in theory, be completely cleared of urea, the more efficient the kidneys. An analogy may make the conception more evident. Suppose a man were set to remove eggs from baskets passing in front of him on an endless chain and that each basket contained 100 eggs, it would be immaterial whether he actually cleared ten eggs out of each of ten baskets per minute, or whether he completely cleared one basket of 100 eggs per minute, his efficiency would be the same in both cases, judging from the number of eggs, 100, that he removed per minute.

Van Slyke and his co-workers showed by direct experiment that when the urinary volume is relatively large the amount of urea excreted in the urine per minute is directly proportional to the blood urea content. When the urinary volume is small this relation no longer holds. The urinary volume above which this relation does hold was termed the "*augmentation limit*"—augmentation of urinary volume beyond this limit does not increase the rate of urea excretion. In adults it was found that the augmentation limit is about 2 c.c. of urine per minute. Clearly the calculation of the blood urea clearance when the urinary volume is above the augmentation limit, must be different from the calculation when the urinary volume is less than 2 c.c. per minute. When the urinary volume is 2 c.c. or more per minute the clearance is termed "*maximum*" when below 2 c.c. per minute the clearance is termed "*standard*".

Formula for Maximum Clearance or  $C_m$ 

Let the volume of urine excreted per minute be  $V$  c c, and the concentration of urea in this urine be  $U$  mgm per 1 c c. Then in one minute  $U \times V$  mgm of urea are excreted, and the same amount must be removed from the blood per minute.

Suppose the blood contains  $B$  mgm of urea per 1 c c. Therefore 1 mgm of urea is contained in  $\frac{1}{B}$  c c of blood, and therefore  $U \times V$  mgm of urea are contained in  $\frac{U \times V}{B}$  c c of blood. Therefore  $\frac{U \times V}{B}$  c c of blood are completely cleared of urea per minute.

$$C_m^x = \frac{U \times V}{B} \text{ c c of blood per minute}$$

Formula for Standard Clearance or  $C_s$ 

Below the augmentation limit it was found by observations on men that the rate of urea excretion varied, on the average, with the square root of the urinary volume. It is therefore necessary to fix the urinary volume at a definite standard, which is not practicable, or to calculate with the aid of the square root law from the observed urea excretion what would be the urea excretion with such a standard urinary volume. As a standard, 1 c c per minute was adopted, which corresponds to 1,440 c c per twenty four hours, a figure very close to the average output by adults per diem.

Suppose  $C$  is the observed blood urea clearance when the urinary volume is  $V$  c c per minute. Let  $C_s$  be the standard clearance with a corresponding standard urinary volume of  $V_s$  c c per minute.

$$\text{Then} \quad C_s : C :: \sqrt{V_s} : \sqrt{V}$$

$$\text{or} \quad C_s = \frac{C\sqrt{V_s}}{\sqrt{V}}$$

But, as mentioned above, 1 c c per minute was adopted as the standard for urinary volume, i e.,  $V_s = 1$ .

$$\text{Therefore} \quad C_s = \frac{C \times 1}{\sqrt{V}}.$$

But  $C$ , the observed clearance, as has been shown for  $C_m$ , the maximum clearance, is equal to  $\frac{U \times V}{B}$ .

Substituting for  $C$ ,

$$C_s = \frac{U \times V}{B} \times \frac{1}{\sqrt{V}} \quad \text{or} \quad \frac{U \times \sqrt{V}}{B} \text{ c c of blood per minute}$$

## Method of Reporting Results

Van Slyke *et al* found that in health the average maximum clearance was 75 c c of blood per minute, and the average standard

clearance 54 c c of blood per minute. Comparisons between the standard clearance of one subject and the maximum clearance of another subject can be made if the results in both are expressed in the same terms, namely, as percentages of the average normal

Thus  $C_m$  becomes  $\frac{U \times V}{B} \times \frac{100}{75}$  per cent of average normal and

$C_s$  becomes  $\frac{U\sqrt{V}}{B} \times \frac{100}{54}$  per cent of average normal

In making these calculations the urinary volume,  $V$ , must be expressed in cubic centimetres of urine per minute. Provided that  $U$  and  $B$  are expressed in the same terms, it does not matter whether milligrammes per 1 c c, or milligrammes per 100 c c, or grammes per cent, or grammes per litre, are chosen

### Correction Factor for Body Size

Clearly a young child with its smaller kidneys will not excrete urea at the same rate as an adult. In other words, a correction factor must be introduced if comparable results for blood urea clearances are to be obtained in subjects differing greatly in size. It has been shown (see *J Clin Invest*, 1928, 6, 467) that the weight of the kidneys varies in proportion to the body surface. Van Slyke *et al* adopted 1.73 square metres as their standard body surface area, and corrected the urine volume per minute by multiplying it by  $\frac{1.73}{A}$ , where  $A$  equals the surface area of the subject

studied. To determine  $\frac{1.73}{A}$  the subject's height is measured in metres, and the correction factor is read off from Fig. 23 using the scale appropriate for the subject's age. This figure was constructed from medico-actuarial tables and Du Bois' formula for calculating the surface area from the height and weight, the reader is referred to the original paper quoted above for a fuller explanation. The completed formulæ for calculating the clearances therefore read

$$C_m = \frac{U}{B} \times V \times \frac{1.73}{A} \text{ or } \frac{U \times V_{cor}}{B} \text{ c c of blood per minute}$$

$$\text{and } C_s = \frac{U}{B} \sqrt{V \times \frac{1.73}{A}} \text{ or } \frac{U \sqrt{V_{cor}}}{B} \text{ c c of blood per minute}$$

In the great majority of adults no appreciable error will be introduced by omitting the correction for body size. Thus Van Slyke *et al* point out that there will result from this omission a variation of only  $\pm 5$  per cent in the maximum clearance,  $C_m$ , if the height lies between 1.64 and 1.76 metres (65 to 69 in.) and of  $\pm 5$  per cent in the standard clearance,  $C_s$ , for heights between 1.57 and 1.81 metres (62 to 71 in.). Naturally the correction must be introduced for all children, and for adults who are obviously short or tall.

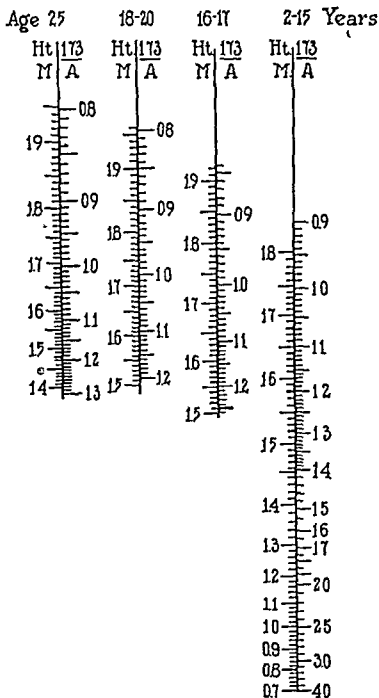


FIG 23 Chart for correction of urinary volume for body size in calculations of blood urea clearances. Multiply the observed volume of urine per minute by  $\frac{1.73}{A}$ . Read the value of  $\frac{1.73}{A}$  opposite to the patient's height in metres, using the scale appropriate for his age. The scale for twenty five years is used also for subjects who are older. (From Peters and Van Slyke's *Quantitative Clinical Chemistry*, Vol II, p 569)

### Preparation of Patient and Collection of Samples

The subject should be at rest in bed (see also below), ordinary meals and fluids are allowed; indeed, to promote a free flow of urine Van Slyke recommends a glass of water at the beginning of the test, and a second glass at the end of one hour.

The urine is collected completely in each of two consecutive and accurately timed one hour periods. Blood is taken at about the middle of the two hours. Thus—

10 a.m. Empty the bladder completely and discard the urine

11 a.m. Empty the bladder completely and send the whole of the specimen to the laboratory

12 noon Repeat as at 11 a.m.

It is not necessary that each period shall be exactly sixty minutes, but it is necessary to know to the nearest minute exactly what are the time intervals (see also below). Two samples of urine are obtained simply to allow of a duplicate determination of the clearance, which serves as a check on the accuracy of collection and the completion of micturition. Just before or just after the first hour's urinary collection, blood is taken. The blood urea is not significantly altered by meals, so that strict adherence to a given time for collection of blood is unnecessary. (For effect of administered urea on the blood urea see later.)

Variations in the times and manner of collecting the urine and blood may be introduced for convenience provided that the urinary volumes are complete, accurately timed and measured. Some subjects find micturition difficult at intervals of only sixty minutes. Alternatively, note the time when urine is voided naturally and discarded and collect the sample at the next natural micturition, again observing the time, and use this sample for the test, collect the blood at some point between the two acts of micturition.

In cases of urinary obstruction catheterisation may be required, but may cause reflex inhibition or reduction of excretion and spoil the test, indeed, in obstructive cases and any others in which the urinary collection is likely to be unsatisfactory, it is wise to give up the clearance test and to substitute the  $\frac{U}{B}$  ratio (see p. 98) after a night's restriction of fluids.

In arriving at the above instructions much work was done by Van Slyke and his colleagues in assessing the possible influence of various factors on the clearance, and this is summarised in the following notes.

**Posture** In normal subjects and in renal diseases accompanied by more than 50 per cent of average normal clearance, there is no loss of accuracy in clearance values determined while the subject is up and about. In more severe renal disease however comparable results are secured only if the subject is at rest in the recumbent position otherwise too low results are obtained. In other words, a preliminary test on an outpatient is permissible but if the result is below 50 per cent of average normal he should be re-tested in bed.

**Diuretics** Drugs such as salyrgan, caffeine and diuretin, and beverages such as coffee and tea, do not affect the clearance values.

• **Salt** Variations in the sodium chloride content of the diet are without effect.

**Protein** Low protein diets, 40 gm. of protein and less daily, may lower the clearance, particularly in health and in mild renal damage, in severe kidney disease this lowering is not so marked or is insignificant.

**Urea.** It is essential that the clearance be not determined during the first hour after 15 gm. of urea, because in that period the blood urea rises rapidly and may be doubled, during the second and third hour, though in health the blood urea is fairly steady at the higher level, in renal disease it may show considerable fluctuations (cf blood urea curves of Archer and Robb). Since the blood is taken once only in clearance tests, it would in theory be wiser to avoid urea administration. On the other hand, the higher blood urea may increase the accuracy of the determination, and the urinary volumes being larger after the draught of urea are more likely to be accompanied by perfect emptying of the bladder.

In very careful work, Van Slyke and his colleagues were unable to show any significant difference in the clearances with and without urea administration. Fowweather believes that the giving of urea prevents the erroneously low clearance occasionally obtained without urea, but Fowweather also reports some clearances with urea which are considerably lower than those without urea. The writer feels that the administration of a solution of 15 gm. of urea may be of practical value in securing more correct urinary volumes, but that water does equally well, he agrees with Van Slyke that urea makes no difference to the true clearance value and is generally an unnecessary complication.

### Analyses

For each sample the urinary volume is measured to the nearest cubic centimetre, and the volume per minute is calculated. The concentrations of urea in the blood and urine are determined by the methods described earlier in this chapter. The hypobromite method (p. 71) for urinary urea is satisfactory, in acidosis it is an advantage that this method includes the nitrogen from ammonia,<sup>1</sup> because the urinary ammonia is formed in the kidney from the urea of the blood (cf p. 184). The blood urea must be determined accurately by an urease method.

#### Example of Calculation of $C_m$

B = blood urea = 55 mgm. per 100 c.c.

#### First Hour

V = urinary volume = 155 c.c. in 62 minutes = 2.5 c.c. per minute

U = urinary urea = 1.06 per cent. = 1,060 mgm. per 100 c.c.

$$C_m = \frac{U \times V}{B} = \frac{1,060 \times 2.5}{55} = 48.2 \text{ c.c. of blood per minute}$$

or  $48.2 \times \frac{100}{75} = 64.3 \text{ per cent. of average normal.}$

#### Second Hour

V = 144 c.c. in 60 minutes = 2.4 c.c. per minute.

U = 1.12 per cent. = 1,120 mgm. per 100 c.c.

$$C_m = \frac{1,120 \times 2.4}{55} = 48.8 \text{ c.c. of blood per minute}$$

or  $48.8 \times \frac{100}{75} = 65.2 \text{ per cent. of average normal.}$

#### Conclusion

Blood urea clearance (maximum) is 64.3 and 65.2, i.e., 65 per cent. of average normal.

<sup>1</sup> Van Slyke finds that more consistent clearances are obtained from "Urea + Ammonia" than from "Urea" alone. If urinary analyses are made by urease methods the preformed ammonia is included and not removed by permutit; "U" then equals 0.95 (Urea + Ammonia, calculated as urea).



Example of Calculation of  $C_u$ 

B = blood urea = 120 mgm per 100 c c

*First Hour*

V = urinary volume = 65 c c in 60 minutes = 1.08 c c per minute

U = urinary urea = 0.90 per cent = 900 mgm per 100 c c

$$C_u = \frac{U\sqrt{V}}{B} = \frac{900\sqrt{1.08}}{120} = \frac{900 \times 1.039}{120} = 7.8 \text{ c c of blood per minute}$$

or  $7.8 \times \frac{100}{54} = 14.4$  per cent of average normal

*Second Hour*

V = 114 c c in 66 minutes = 1.73 c c per minute

U = 0.70 per cent = 700 mgm per 100 c c

$$C_u = \frac{700\sqrt{1.73}}{120} = \frac{700 \times 1.315}{120} = 7.7 \text{ c c of blood per minute}$$

or  $7.7 \times \frac{100}{54} = 14.2$  per cent of average normal

*Conclusion*

Blood urea clearance (standard) is 14.4 and 14.2, i.e., 14 per cent of average normal

Example of  $C_u$  with Correction for Body Size

Child, aged four, height 42 in or 1.07 metres (1 metre = 39.37 in)

The correction factor to be applied to the urinary volumes is read off from the last column (ages two to fifteen) of Fig. 23, it is 2.3

B = blood urea = 54 mgm per 100 c c

*First Hour*

V = urinary volume = 38 c c in 68 minutes

$V_{cor}$  = corrected urinary volume =  $38 \times 2.3 = 87.4$  c c in 68 minutes  
= 1.28 c c per minute

U = urinary urea = 1.70 per cent = 1,700 mgm per 100 c c

$$C_u = \frac{U\sqrt{V_{cor}}}{B} = \frac{1,700 \times \sqrt{1.28}}{54} = \frac{1,700 \times 1.131}{54} = 35.6 \text{ c c of blood per minute}$$

or  $35.6 \times \frac{100}{54} = 65.8$  per cent of average normal

*Second Hour*

V = 46 c c in 97 minutes

$V_{cor}$  =  $46 \times 2.3 = 105.8$  c c in 97 minutes = 1.09 c c per minute

U = 1.55 per cent = 1,550 mgm per 100 c c

$$C_u = \frac{1,550 \times \sqrt{1.09}}{54} = \frac{1,550 \times 1.044}{54} = 30.0 \text{ c c of blood per minute}$$

or  $30.0 \times \frac{100}{54} = 55.6$  per cent of average normal

### Conclusion

Blood urea clearance (standard corrected for size) is 65.8 and 55.6, i.e., 61 per cent of average normal

### Interpretation of Clearance Tests

The danger of expressing the results of a biological test in mathematical terms is that the reader may attach undue significance to small variations. This danger is minimised, however, if it be remembered that such tests usually show a variation of the order of  $\pm 20$  per cent. The blood urea clearance test is no exception, for Van Slyke *et al* derived their average maximum clearance of 75 c.c. of blood per minute from figures ranging from 60 to 95 c.c., and their average standard clearance of 54 c.c. of blood per minute from figures ranging from 40 to 65 c.c.

Obviously clearances above 100 per cent of average normal clinically are satisfactory. The important point is what figure shall be taken as the lower limit of the range in health, the majority of workers have agreed that 70 per cent of average normal represents this lower limit. Dr Van Slyke, in a personal communication to the writer in 1934 kindly made the following suggestions for interpreting the results of clearance tests —

Percentage of Average Normal Clearance	Renal Function
Over 70	Normal
70 to 40	Mild deficit
40 to 20	Moderate deficit
Below 20	Severe deficit
Below 5	Uræmic coma present or imminent

This scale agrees well with the results obtained in the writer's laboratory.

The clearance value varies with the flow of blood through the kidneys (Van Slyke). It is not constant in a given healthy individual, but varies within narrow limits in accordance with the normal functional elasticity of the kidneys. In severe renal disease it is lowered and more fixed. The effect of renal blood flow should be remembered, for the clearance value, like the blood urea (*cf* p 83), is altered by non renal factors which influence the oxygen supply, circulation and filtration pressure in the kidneys, viz., severe anæmia and anhydræmia, the poor circulation of cardiac decompensation, etc.

### DYE TESTS

#### Indigo-Carmine

This test is used extensively in surgical work, and almost invariably by the surgeon himself. For a fuller description the reader is referred to books on urinogenital surgery.

The dye is injected intravenously in amounts varying between 0.08 and 0.16 gm (20 c.c. of 0.4 per cent solution = 0.08 gm). Either the combined urine or the segregated urines (obtained by ureteric catheterisation) are then examined. The points to be noted are, the time elapsing between injection and the first appearance of the dye, the time of maximum colour, and the time required for complete excretion. The indigo carmine test is, therefore, a "time" test. Quantitative colorimetric determinations are not satisfactory (cf p. 229). It is used particularly in diagnosing which is the kidney affected, or in obtaining a rough comparison between the efficiency of each of the two kidneys. The following table may be useful in interpreting the surgeon's report.

	Normal	Abnormal
Appearance of colour	5 to 20 minutes	Delayed beyond 20 minutes
Maximum colour	About 45 minutes	After 1 hour
Total excretion	Complete in 14 hours (greater portion in 12 hours)	Delayed beyond 14 hours

### ✓ Phenol Red or Phenolsulphonephthalein

This dye is used both as a "time" test (in the same way as indigo carmine) and as a quantitative test.

Injections are made subcutaneously, intramuscularly or intravenously. The usual amount is 6 mgm (0.006 gm in 1 c.c.).

*As a "Time" Test* Before the test the patient drinks enough water (about 300 c.c. or 10 oz.) to ensure a good flow of urine. On completion of catheterisation the dye is injected, and the urine is received in a test tube containing a few drops of liquor potassæ or caustic soda solution (10 to 25 per cent.)

	Normal	Abnormal
✓ Appearance of colour	5 to 10 minutes	Delayed beyond 10 minutes
✓ Maximum colour	15 to 20 minutes	Delayed beyond 20 minutes
✓ Total excretion	Complete in 4 hours (greater portion in 2 hours)	Delayed beyond 4 hours

*As a Quantitative Test* The patient is instructed to empty the bladder completely (specimen 0) and to drink about  $\frac{1}{2}$  pint of water. The 6 mgm. of dye are then injected. The urine is collected at the end of one hour and again at the end of two hours after the injection.

The quantity of dye in each of these specimens is estimated colorimetrically. For this purpose a quantity of a 25 per cent solution of caustic soda is added sufficient to develop the maximum red colour. The mixture is then diluted to 1,000 c c. As a standard 3 mgm of phenol red (0.5 c c of standard solution of dye) are added to some of the urine obtained *before* the injection (specimen 0). The volume of specimen 0 used is made approximately equal to the volume passed during the second hour. NaOH (25 per cent) is then added to develop the maximum colour, and the mixture is diluted to 1,000 c c. The use of specimen 0 in preparing the standard compensates very largely for the difference in tints often observed between the treated urine and the more usual standard made up with water, but no urine.

The unknowns and the standard are allowed to stand till the precipitate of phosphates settles, or they are filtered. The supernatant fluids or filtrates are then compared in a colorimeter. The percentage of the dye excreted in the first and in the second hour is then calculated. If blood be present the urine is made alkaline by adding powdered lime,  $\text{Ca}(\text{OH})_2$ , or milk of lime (slaked lime made into a thin cream with water), instead of the 25 per cent NaOH. On standing, or after filtration, the blood pigments are thereby removed as well as the phosphates.

Normally 40 to 60 per cent of the dye is recovered at the end of the first hour, and 15 to 25 per cent at the end of the second hour. The total recovery in the two hours normally varies from 60 to 85 per cent. Figures below 40 per cent (first hour) or 60 per cent (two hours combined) suggest renal inefficiency. It should be noted that children excrete phenol red better than adults. In two hours normal children excrete 70 per cent or more.

The phenol red test is a measure of the power of the kidneys to excrete a foreign substance. It has been very extensively used in America and to a less extent in this country. The general consensus of opinion is that it runs parallel with the blood urea. It is subject to practically the same limitations as the latter, when used as a quantitative test.

## ✓ CHLORIDE CONTENT OF BLOOD AND URINE

So far all the methods considered have been designed to test the efficiency of the excretory function of the kidneys. Methods, aiming at an estimation of the power of the kidneys to maintain the osmotic pressure of the blood at a steady level are frankly disappointing. The chief reason for this is that the blood chlorides, and particularly the excretion of chlorides, are frequently affected by purely non renal factors.

The chloride content of the urine is very largely dependent on the diet, so that quantitative estimations of output are of very little value unless combined with estimations of the intake. Even on a constant intake of salt it apparently takes several days for a balance between input and output to be established. In short,

urinary chlorides are only mentioned in order to warn the reader that their estimation is of little or no value as a measure of renal efficiency. It is, of course, easy enough to show by simple qualitative tests whether or no the concentration of the urinary chlorides is diminished, but how often is such a diminution due to renal inadequacy? In nephritis with œdema there is obviously chloride retention, but often the more potent factor in reducing the urinary chlorides is restriction of salt in the diet. Perhaps an example will make it clearer. Two cases (A and B) of nephritis with œdema excrete respectively 1 and 5 gm of chlorides (calculated as NaCl) in twenty four hours. From this result we have no right to infer that A's kidneys are less efficient than those of B. A may be taking less salt than B.

De Wesselow claimed good results in clinical work from an estimation of the *concentration* of chlorides in the urine after a provocative dose of 4 gm of potassium chloride. However, as in tests with sodium chloride, Leiter found that the results depended greatly on the amount of chloride previously taken in the diet. With KCl there is little or no risk of seriously increasing the œdema in cases already œdematous as there was with some of the older NaCl tests. The capacity of the kidney for chloride concentration appears to run parallel with its power of concentrating urea. An apparent dissociation of the two functions is seen in cases of parenchymatous nephritis with gross anasarca, but this would appear to be due to impoverishment of the blood in chloride. It may be suggested that the kidneys in chronic parenchymatous nephritis lack the opportunity rather than the capacity for excreting chloride. The œdema is possibly extrarenal in origin, it may be due to an alteration in the permeability of the capillaries generally. Alternatively it has been suggested that it is due to excessive reabsorption by the renal tubules (Dunn).

Estimations of the concentration of chlorides in the blood are disappointing. When chlorides are retained water is also retained, so that the *concentration* of chlorides is little affected. The effect of retention of water plus chlorides is œdema, so that for clinical purposes the degree of œdema is the best measure of chloride retention.

For a further discussion of blood chlorides the reader is referred to Chapter XIX.

## CO<sub>2</sub> COMBINING POWER OF PLASMA

(Alkali reserve plasma bicarbonate)

The third function of the kidney in providing for the fine adjustment of the reaction of the blood is again a difficult function to assess. The kidney is only one of the factors in this adjustment and, therefore, when a deficiency in alkali reserve is demonstrated the kidney may or may not be damaged. In other words, the plasma bicarbonate is of no value in the diagnosis of renal lesions but in known cases of kidney disease its estimation is valuable in

assessing the risks of acidosis. Normally, the alkali reserve corresponds to 53 to 77 c c of  $\text{CO}_2$  per 100 c c of plasma.

Damage to the kidneys may be the direct cause of acidosis by impairing the excretion of phosphates and sulphates. Also in severe renal disease the kidneys may be unable to excrete ammonia, or, in view of the work of Nash and Benedict, it may be more correct to suppose that the badly damaged kidney fails to change urea into ammonium carbonate. The result of each of these failings would be the fixation of a larger proportion of plasma bases than normal, leaving less to be combined as bicarbonate.

For further discussion the reader is referred to Chapter IX.

### BLOOD CHOLESTEROL

Normally cholesterol is not excreted by the kidneys, or, at any rate, not in amounts exceeding 3 to 4 mgm per diem (Gardner and Gainsborough). Practically all the excretion of cholesterol is performed by the liver, *via* the bile. In parenchymatous nephritis with marked proteinuria quite appreciable quantities of cholesterol may be excreted in the urine, possibly in combination with globulin ('lipoid globulin'). There is no evidence that the kidneys have any *direct* effect on the concentration of cholesterol in the blood. None the less, in certain renal conditions estimations of the blood

#### *Blood Cholesterol and Urea in Nephritis*

Disease	Age Years	No of Cases	mgm per 100 c c of whole blood					
			Cholesterol			Urea		
			Lowest	High est	Ave- rage	Low est	High est	Ave- rage
Normal *	5½-13	38	105	214	165	21	58	38
Acute nephritis	1-10	18	131	315	182	28	255	67
Hæmorrhagic nephritis	4-11	9	126	231	166	28	85	52
Chronic azotæmic nephritis (chronic interstitial nephritis) †	1-11	11	114	294	193	41	692	289
Mixed azotæmic and hydræmic nephritis	2-15	7	154	810	316	53	297	112
Chronic hydræmic nephritis (chr parenchymatous nephritis or nephrosis)	1-8	9	279	791	479	20	31	26

\* Many of these were convalescent surgical cases. The rest were medical cases excluding renal diseases and all conditions in which hypercholesterolaemia might be expected (e.g. diabetes mellitus, hepatic lesions, xanthomatosis, etc.)

† Most of these were renal dwarfs.

cholesterol are interesting (*cf* table, p 95) Thus, in nephritis with oedema the blood cholesterol is often raised It has been suggested that hypercholesterolaemia in these cases is due to an attempt to neutralise toxins, cholesterol having antitoxic properties It is possible that owing to failure on the part of the kidney to excrete toxins, the cholesterol mechanism is called into play However that may be, estimations of blood cholesterol are sometimes useful in the diagnosis of chronic parenchymatous nephritis, and occasionally as a guide to prognosis and treatment in that condition, though it must be admitted that as a general rule such estimations are superfluous Normally the cholesterol content of whole blood is 100 to 200, and of plasma or serum 100 to 220 mgm per 100 c.c. In chronic parenchymatous nephritis the figures exceed 200 mgm, and may occasionally be as high as 800 mgm As the patient improves the figures tend to return to normal

For further discussion of conditions influencing cholesterolaemia the reader is referred to Chapter XIX

### ✓ PLASMA PROTEINS AND SERUM CALCIUM

These are considered more fully in Chapter XIX, the most striking alterations in kidney disease alone will be noted here Like blood cholesterol determinations, they are of academic interest rather than of practical value In nephritis with oedema (nephrosis especially) the total protein of the plasma falls, and this fall is limited to the albumin fraction That the proportion of albumin in the urine is at the same time increased was noted previously (p 26) as also that serum albumin has a smaller molecule than serum globulin (table p 27) The globulins of the plasma do not fall (an increase has at times been reported), with the result that there is a rise in the globulin expressed as a percentage of the total protein

Moore and Van Slyke have shown that there is a critical level—about 2.5 per cent albumin and about 5.5 per cent total protein—above which oedema commonly does not, and below which oedema commonly does occur in untreated Bright's disease

The serum calcium may be lowered in nephritis, but tetany is seldom noted The hypocalcaemia is associated either with low serum proteins—probably due to a coincident fall in that portion of the calcium which is combined with the serum proteins—as in nephrosis, or with an increase in the inorganic phosphate (p 101) as in azotæmic nephritis The low calcium may be one factor in the causation of uræmic symptoms

### INDICANÆMIA

✓ I Diazo Test (Andrewes' Reaction) Andrewes noted that uræmic sera gave a peculiar buff colour when Van den Bergh's indirect test (see Chapter XII) was performed Further, and this is the characteristic part of the reaction, when NaOH was added the colour changed to pink or cherry red (Azobilirubin, on the other hand, turns from pink to green on the addition of alkali) Hewitt

studied this reaction and introduced a slight modification, thereby making the test simple and rapid, and suitable for clinical purposes. The reaction is due to indican (Harrison and Bromfield). Marked indicanæmia is found only in severe renal inefficiency. Large quantities of indoxyl compounds may be excreted in the urine in many diseases (see Chapter XIV), but the concentration in the blood does not rise appreciably if the kidneys are efficient. If the test is positive then renal inefficiency is indicated, but a negative result does not exclude uræmia. Such negative results in uræmia are, however, rare, and may possibly be explained by lack of formation of indican. Indoxyl is formed from tryptophan by bacterial decomposition in the intestine (see Chapter XIV), and is then conjugated in the liver either with sulphate to form potassium indoxyl sulphate (indican) or with glycuronic acid. The possible factors influencing the formation of indican are, therefore, the intake of protein containing tryptophan, the type of intestinal flora, and the functional efficiency of the liver. It is easy to imagine that negative Andrewes' tests in uræmia are accounted for by variations in one or other of these factors.

**Technique** (see Harrison and Hewitt) **Reagents** (identical with those used in Van den Bergh's test)

**Solution A**

Sulphanilic acid	1 gm
Concentrated hydrochloric acid	15 c c
Distilled water	to 1,000 "

**Solution B**

Sodium nitrite	0.5 gm
Distilled water	to 100 c c

The diazo reagent is prepared by mixing 10 c c of solution A with 0.3 c c of solution B. The mixture may be kept for at least a week at room temperature without loss of sensitivity so far as Andrewes' reaction is concerned, although, of course the solutions A and B must be mixed freshly for use in Van den Bergh's test. The mixture often turns slightly yellow after keeping for a day or two, but this does not matter in Andrewes' test.

To 1 volume of serum 2 volumes of alcohol (absolute or 96 per cent) are added. The precipitated proteins are separated by centrifuging or filtering. (A slightly improved extraction may be obtained by heating the mixture of serum and alcohol to the boiling point.) To 4 volumes of the filtrate 1 volume of the diazo reagent is added. The mixture is boiled thoroughly for half to one minute, and cooled. A solution of 40 per cent caustic soda is added drop by drop<sup>1</sup> shaking after each addition.

The colour change on making alkaline with NaOH is the essential part of the reaction. The test should be called positive only when a definite pink or cherry red colour is seen. This pink colour is fleeting. It may last for a few seconds only, and the mixture must,

<sup>1</sup> One drop of 40 per cent NaOH is ample for 2 c c of filtrate plus 0.5 c c of diazo reagent.



therefore, be observed carefully whilst adding the NaOH. In a few very severe cases of uræmia the pink colour may persist for longer periods, even up to half an hour. The test in its present form is only qualitative. It has been found that the volumes recommended may be judged sufficiently well by eye without greatly lessening the sensitivity of the reaction.

**II. Oxidation to Indigo Blue** The quantity of indican in plasma, even in severe renal inefficiency, is so small that careful technique is necessary to obtain positive results. The following application of Jolles' test by Bolliger and Earlam is recommended, it is definitely more sensitive than the diazo test.

To 2 c.c. of plasma (or serum) add 2 c.c. of 20 per cent trichloroacetic acid, mix and centrifuge. To 2 c.c. of the supernatant fluid add 1 c.c. of 5 per cent thymol in alcohol, and 10 c.c. of Obermayer's reagent (see Appendix), mix well and set aside for twenty minutes. Then add 2 c.c. of chloroform mix well and allow the chloroform layer to separate, or centrifuge. Depending on the amount of indican, the chloroform may be deep lavender at once (+++), pale lavender at once (++), or show a pale lavender colour only after standing overnight (+), or be colourless immediately and next morning (0).

## NOTES ON OTHER TESTS OF RENAL EFFICIENCY

**Urea Concentration Factor** The urea contents of blood and of urine obtained simultaneously are estimated, and the ratio

$$\frac{\text{milligrammes of urea per 100 c.c. of urine}}{\text{milligrammes of urea per 100 c.c. of blood}} \quad \text{or} \quad \frac{U}{B}$$

is calculated. MacLean determined this ratio under ordinary conditions of diet, etc. In his experience factors of 60 to 80 were normal, 60 to 20 doubtful, and less than 20 dangerous. Harrison calculated the factor from estimations made in the third hour of the urea concentration test, i.e., after 15 gm. of urea. By this means the determination was made under more standard conditions, the results in health varied from 40 to 80, Rabinowitch and others confirmed its value. Van Slyke and his colleagues pointed out that this method of determining the urea concentration factor was essentially the same as determining the standard urea clearance, it was the fact that fluids were withheld in the urea concentration test (cf p. 70), and not the giving of 15 gm. of urea, that made

this  $\frac{U}{B}$  ratio more satisfactory. Provided that the urine volume is kept within ordinary limits, less than 2 c.c. per minute and as near as possible to 1 c.c. per minute the  $\frac{U}{B}$  ratio may be regarded as a useful substitute for the standard urea clearance test, if fluids are withheld overnight, this ratio may be of more practical assistance than attempts to estimate the clearance in patients with retention or incontinence of urine, or in subjects who have difficulty in

completely evacuating the bladder or in micturating at fixed intervals (cf. remarks on blood urea clearance test, p. 88).

**Blood Non-protein Nitrogen.** The non-protein nitrogen of the blood, as the name implies, includes all compounds of nitrogen other than proteins (i.e., all nitrogen which is "non-protein" at the time of the analysis; its previous origin from proteins or otherwise is immaterial). Thus the nitrogen of urea, uric acid, creatinine, creatine, amino-acids and other substances ("rest nitrogen") is included. The urea nitrogen in health generally constitutes from approximately one-third to slightly over one-half of the total non-protein nitrogen of the blood.<sup>1</sup> When, however, the non-protein nitrogen is markedly raised, the urea nitrogen

*Showing Increase in the Proportion of the Urea Nitrogen with Increasing Retention of Non-protein Nitrogen in the Blood in Kidney Diseases*

Case No	mgm per 100 c.c. of whole blood			Urea nitrogen as percentage of non protein nitrogen
	N P N	Urea	Urea nitrogen	
1	374	585	273	73
2	342	592	276	81
3	278	501	234	84
4	166	297	139	84
5	141	237	111	78
6	96	154	72	75
7	73	98	46	63
8	60	85	40	67
9	60	67	31	52
10	54	52	24	45
11	42	39	18	43
12	37	29	14	37

*Notes* Case 1 was an adult, the remaining cases children in the Hospital for Sick Children, Great Ormond Street

Most of the patients had chronic interstitial nephritis

<sup>1</sup> Contrast this with the condition existing in the urine, in which the urea nitrogen generally constitutes a much higher proportion of the total (non protein) nitrogen (80 to 90 per cent. on an average mixed diet)

constitutes a higher proportion of the total, and may even exceed 80 per cent (*cf* table, p 99)

In other words, as the kidney becomes very grossly damaged the nitrogen partition of the blood approaches that of normal urine, the kidney loses its excretory power. An estimation of the blood urea is simpler than an estimation of the non protein nitrogen, and is, therefore, generally preferred as a routine measure. Some writers claim, however, that the non protein nitrogen is of value because it is definitely raised in those cases of nitrogen retention in which the blood urea has been artificially reduced by low protein diets.

Normally the non protein nitrogen of the blood varies from 25 to 50 mgm per 100 c c (see Chapter XIX)

**Blood Creatinine** Definite retention of creatinine in the blood only occurs when the kidney damage is gross. Estimations of creatinine, therefore, are not of as much value as estimations of urea for diagnostic purposes. But several writers have claimed that the creatinine determinations are of value in prognosis. Values exceeding 5 mgm per 100 c c are considered indicative of a very serious prognosis in chronic renal lesions, the great majority of such cases dying within a few months.

Normally the blood creatinine lies between 0.7 and 2 mgm per 100 c c but occasionally figures as high as 3.5 mgm are encountered in hospital patients in whom there is no evidence of renal disease. Creatinine is distributed in approximately equal amounts between corpuscles and plasma.

**Urinary Creatinine** Estimations of the concentration of creatinine in the urine or of the total output in twenty four hours are of little or no value as measures of renal efficiency, but the excretion hourly or at intervals of fifteen minutes after  $\frac{1}{2}$  gm of creatinine intravenously is regarded as valuable by Major.

**Blood Uric Acid** It has been claimed that uric acid is the first of the nitrogenous bodies to be retained in azotæmic nephritis, and that, therefore, it is of value in the diagnosis of nephritis in the early stages as well as in the last stages in which there is, of course a general retention of nitrogenous waste products. Urea is the next to be retained and creatinine the last. Other workers, however, do not believe in the existence of this "staircase" effect. They consider that when a failure of nitrogenous excretion sets in all three compounds are retained simultaneously.

Normally the amount of uric acid in the blood varies from 0.3 to 4 mgm, and usually lies between 1 and 3 mgm per 100 c c. It is slightly higher in the first three or four days of life. Thus most of the figures of Kingsbury and Sedgwick lie between 3 and 5 mgm for the first four days of life. At the end of the first week the blood uric acid usually lies between 1 and 3 mgm.

Whether or not the increased uric acid content of the blood in gout is due to retention by the kidneys is still not settled. For further discussion see Chapter XIX.

**Diastase Tests** The concentration of diastase (amylase) in the

urine is often subnormal in renal disease, but a low urinary diastase is not characteristic of kidney lesions. The excretion of amylase may be subnormal simply owing to a diminished production of the ferment in the body. If a diminished excretion is combined with a retention of diastase in the blood, severe renal disease may safely be inferred. The estimation of diastase in blood and urine collected simultaneously (see Stafford and Addis, and Harrison and Lawrence) is not a delicate test of renal inefficiency, and has been almost universally discarded.

**Sugar Content of Blood and Urine** In a few cases of renal disease the threshold for glucose is lowered, causing one type of renal glycosuria. In a fair number it is raised, causing hyperglycæmia without glycosuria, but a raised threshold occurs in other conditions without associated kidney damage (notably in diabetes mellitus). In view of these points, and the fact that the level of blood sugar is largely controlled by other non renal factors (Chapter VII), sugar tests are generally regarded as useless as measures of renal efficiency.

**Inorganic Phosphates in Plasma or Serum** Retention of inorganic phosphates runs roughly parallel with retention of nitrogenous bodies, though de Wesselow finds the former more valuable in the prognosis of the azotæmic type of nephritis. He considers that a figure above 10 mgm per 100 cc almost invariably indicates a hopeless prognosis. This retention of phosphates is one of the factors causing acidosis in nephritis.

## REFERENCES TO FURTHER TESTS

In addition to the general accounts of tests of renal efficiency, given in the books mentioned at the beginning of this chapter, the following will be found of interest —

*Water Meals*, combined with other tests

LEATHES J B *Brit Med J* 1919 ii, 165, and *Lancet*, 1920, ii, 933

CALVERT E G B *Brit Med J* 1925, i, 64

*Mosenthal's Test*

MOSENTHAL, H O *Arch Int Med* 1915, 16, 733

MOSENTHAL, H O, and LEWIS D S *J Amer Med Assoc*, 1916 67, 933

*Addis Ratio*

ADDIS T *Arch Int Med*, 1922 30, 378

*Excretion of Potassium Iodide*

ROWNTREE L G and FITZ, R *Arch Int Med*, 1913 11, 258

LINDER G C *Quart J Med*, 1922 15, 227

*Excretion of Lactose*

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*Synthesis of Hippuric Acid from Benzoic Acid (Benzoates)*

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KINGSBURY, F B *Arch Int Med*, 1923 32, 175

*Alkaline Tide*

LEATHES J B *Brit Med J* 1919 ii, 165, and *Lancet* 1920, ii, 933

BRUNTON, C E *J Physiol* 1933, 78 65

## SELECTION AND VALUE OF TESTS IN MEDICAL AND SURGICAL WORK

It is difficult to give advice as to the selection of tests which shall be of general application, because at one centre certain tests are used more than others, and because it is not feasible for any one individual to gain extensive experience of all the tests that have been reported to be of genuine value. In clinical work preference will naturally be shown for the simpler tests. At the same time, as noted above (p 70), the urea tests are probably more widely used than any others, and by many clinicians almost exclusively.

### Emergencies

A patient is unconscious, and a tentative diagnosis of uræmia is made. An estimation of urea in the blood or cerebrospinal fluid may often settle the diagnosis. A figure of over 200 mgm per 100 c c may be regarded as pathognomonic, as also would be a positive finding by a test for indicanæmia. A blood urea between 100 and 200 mgm would likewise be almost definitely in support of uræmia, but the significance of these, and certainly of figures between 50 and 100 mgm, should be established by estimating the urea concentration in the urine. An isolated specimen of urine will do for this purpose, catheterisation often being necessary. If a high blood urea is accompanied by a high concentration of urea in the urine, the raised value is probably due to a poor or failing circulation, anhydræmia, etc., and is not indicative of renal failure. If, on the contrary the urea concentration in the urine is low, the urea retention may safely be ascribed to renal damage. In the former case the urinary urea often exceeds 3 or even 4 per cent; in the latter it is generally less than 2 per cent. It is particularly important to remember possible fallacies in interpreting blood urea findings in any moribund or gravely ill patient.

### Medical Cases

Urea tests should be performed as a routine in order to obtain a measure of the degree of damage to function. As a rule the urea concentration test should first be performed. If the maximum exceeds 2.5 per cent, little additional information will be obtained from an estimation of the urea in the blood. If the urinary urea is less than 2.5 per cent, resort should also be made to blood analysis. If, for one reason or another, blood analysis is made in the first instance, then this should be checked by the urea concentration test, unless urea retention is gross, in which case the urinary test is superfluous. Nowadays the estimations in blood and urine are often automatically combined in the "blood urea clearance test", where that is the case the urea concentration test is generally omitted.

In the acute stage of nephritis urea retention does not have the same serious import as in the chronic. The blood urea frequently

falls as the acute process subsides. The course of the disease may be followed by repetition of the tests at intervals of a week to a month. A persistently elevated blood urea is of serious import. In chronic nephritis, without œdema, urea tests are invaluable in assessing roughly the amount of damage to function, and as a guide to progress and treatment. Occasionally a gross excess of urea in the blood is the first warning of impending uræmia. It may thus alter completely the clinical opinion. The following is an example — A little girl, age nine, undersized, had thirst and polyuria with slight proteinuria and a few casts. She was up and about the ward when blood was taken for urea estimation purely as a matter of routine. To everyone's astonishment her blood contained 692 mgm per 100 c.c. She was at once sent to bed, and next day she vomited and complained of headache for the first time. She died from uræmia in three days. In chronic nephritis with œdema, nitrogenous (urea) retention accompanies chloride retention in the majority of adults, but in children purely chloride retention is much the commoner. Urea tests are valuable when selecting œdematous patients for treatment with urea or by Epstein's diet. The reactions for indicanæmia are simple and are positive only in gross renal inadequacy. A negative test does not, however, exclude uræmia absolutely. Cholesterol estimations though generally superfluous, are occasionally useful in nephritis with œdema in differentiating the type of nephritis (see table on p. 95), and as a guide to treatment. Determinations of the plasma bicarbonate are valuable in detecting acidosis in nephritis, and in controlling the treatment of that condition by alkali.

In cases of high blood pressure the urea tests are valuable in separating those in which extensive renal damage has occurred. The prognosis is worse in this latter class. The tests do not differentiate nephritis which is primary from nephritis which is secondary to hypertension.

In other medical conditions, pyelonephritis, pyelitis, congenital cystic disease, renal dwarfism, etc., etc., a selection of tests is made on the general lines given above.

### Surgical Cases

In conjunction with ureteric catheterisation the surgeon finds the dye tests invaluable in localising the side affected in stone, tuberculosis, tumour of the kidneys etc., or in coming to an opinion as to the condition of the "sound side" before performing a nephrectomy. Of the dye tests the phenol red test alone lends itself to quantitative determinations. Urea tests in the segregated urines are similarly of value, in fact, comparative estimations of a number of substances, potassium iodide, lactose, diastase, etc., etc., are equally of value, but the urea tests are perhaps the simplest. Blood analysis and tests on the "combined urine" are of value in the same way as in medical cases, in fact, the division into medical and surgical groups is largely an artificial one.

A large group of surgical cases is characterised by obstruction, and of this group cases of enlarged prostate are by far the most numerous. Here, again, the urea tests are the most frequently employed, but the part played by obstruction rather alters the interpretation of the results. It is common for the retained products in the blood to fall rapidly after relief of the obstruction, and an initially raised blood urea does not generally have the same serious significance as in the medical group. None the less the degree of urea retention is of great assistance to the surgeon in deciding whether he shall operate in one or two stages in cases of enlarged prostate. Of course, the urea tests are an extension of the clinical examination, and the meaning of the results must be arrived at in conjunction with the clinical data. For this reason one must be wary in making any "rules," but many surgeons from experience generally prefer a two stage operation whenever the initial blood urea exceeds 100 mgm per 100 c c. The blood urea clearance test is often unsatisfactory in prostatic and other obstructive cases owing to the difficulties of accurately collecting urine at measured intervals of time. The  $\frac{U}{B}$  ratio (see p 98), determined after abstention from fluid overnight, is more practicable.

### Obstetric Cases

Renal efficiency tests are rarely of much value in pregnancy. The blood urea is lower in the later months of pregnancy than in the non gravid. For this reason values above 40 mgm per 100 c c may be regarded as definitely pathological. The chief value of the urea tests is in helping to differentiate chronic nephritis complicated by pregnancy from the toxæmias of pregnancy. In the latter group the urea results very commonly fall within the normal limits for the pregnant woman.

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## CHAPTER VI

### REDUCING SUBSTANCES IN THE URINE—GLYCOSURIA, LACTOSURIA, PENTOSURIA, etc.

**Books** The reader will find the subject discussed in most books of chemical physiology and of clinical diagnosis. For a review of sugars other than glucose, see Garrod, A. E., *Quart J Med*, 1909, 2, 438. The following account is an attempt to face the problems in the way they are most commonly met with in clinical work. For a full and interesting description of pentosuria and alkaptonuria reference should be made to Garrod's *Inborn Errors of Metabolism*. The whole question of glycosuria and the significance of the other reducing substances is discussed in detail in works on diabetes mellitus, e.g., Joslin's *Treatment of Diabetes Mellitus*, Allen's *Glycosuria and Diabetes*, Graham's *Pathology and Treatment of Diabetes Mellitus*, etc.

WE have seen how the detection of protein in the urine should be followed by an examination of the urinary deposit, and have reviewed some of the renal efficiency tests that may be used to extend further the examination of proteinurics. We now turn to the problems that follow the detection of "sugar" (reducing substances) in the urine.

Many substances besides glucose reduce cupric solutions. As a practical working rule, if the amount of reducing substance is considerable and if there are typical symptoms of diabetes mellitus, it may safely be assumed that the reducing substance is glucose. In all other cases additional tests should be performed. Very commonly the next step taken is an estimation of the blood sugar (Chapters VII and VIII). If there is obvious hyperglycæmia (blood sugar above 200 mgm per 100 c.c.), it is fairly safe to assume that the reducing substance is glucose. In the absence of obvious hyperglycæmia the nature of the urinary reducing substance should always be determined.

#### QUALITATIVE TESTS FOR REDUCING SUBSTANCES ("SUGAR")

A large number of tests have been devised for detecting the reducing substances which may occur in urine; but in clinical medicine only two are commonly used, viz., Benedict's and Fehling's tests. Reference is also made below to Nylander's test.

##### Benedict's Test (Qualitative)

Cupric sulphate in alkaline solution is reduced to cuprous oxide by boiling with reducing agents. Although intermediate reduction

products in the form of green precipitates may occur, it is a sound practical rule to ignore these, and to regard Benedict's test as positive only when a yellow or red precipitate of cuprous oxide is seen. It may be necessary, when the quantity of reducing substance is small, to wait for ten to fifteen minutes after the boiling to allow the mixture to cool and the precipitate to settle to the bottom of the tube. There is then no difficulty in deciding whether or no a yellow precipitate has formed. A white or greyish white precipitate is of no significance, since it is due to the earthy phosphates in the urine.

Benedict's reagent will detect "sugar" in urine when it amounts to 0.15 per cent or higher. There are traces of "sugar" in normal urine, but not sufficient to reduce Benedict's reagent. About 1 gm. of reducing substance is excreted in the twenty-four hours by a normal person, but the concentration in any given specimen rarely exceeds 0.1 per cent. When the urine is very concentrated, Benedict's reagent may be reduced by uric acid and creatinine.

Benedict's solution is more sensitive than Fehling's, and, at the same time, is less likely to be reduced by normal urinary ingredients. It is therefore, to be preferred for routine use. Benedict's *quantitative* solution has a different composition from the *qualitative* reagent, and when reduced yields a white instead of a yellow precipitate. This is noted here because the writer has met with instances in which glycosuria was missed owing to the employment of the quantitative reagent for qualitative tests.

In Benedict's test it is recommended that 8 drops of urine be added to 5 c.c. of the reagent. Seeing that the size of a drop varies with the external diameter of the tip of the pipette used, and also on several other factors, either the volumes of the urine and of the reagent should be measured quantitatively, or the volumes of both should be determined approximately as detailed in Chapter II. For careful work it is recommended that 5 c.c. of reagent be heated with 0.5 c.c. of urine in a boiling water bath for five minutes. Under these conditions the Benedict's solution is completely decolorised when the urine contains 2 per cent or more of reducing substance (calculated as glucose).

### Fehling's Test

The principle in this test is the same as in that of Benedict, cupric sulphate being reduced to cuprous oxide. The copper solution, however, is made up with stronger alkali. For that reason small quantities of sugar are more likely to be caramelised, and so rendered ineffective as reducing agents. Hence Fehling's solution is not so delicate as Benedict's. Moreover, Fehling's reagent is more apt to yield greenish precipitates, owing to the action of the stronger alkali on normal urinary ingredients. In connection with these greenish tints, however, it should be remembered that, from a purely clinical point of view, they occur only when the amount of reducing substance is very small, and,

therefore, they may generally be disregarded. There is one advantage in favour of Fehling's solution owing to the fact that it is a less delicate test, it may more conveniently be used as a rough measure of the quantity of sugar when such is present in moderate or large amounts (see later).

When testing with either Benedict's or Fehling's solution, a *large amount of protein may cause trouble, owing to the precipitate of coagulated protein on boiling*. In fact, the decomposition products of the protein may actually reduce the cupric solutions if boiling be prolonged. The difficulty is easily overcome by coagulating the proteins in the original urine, filtering and testing the protein free filtrate for sugar. In urines containing small or moderate amounts of protein no difficulty is experienced, and removal of protein is unnecessary.

For biuret reactions at room temperature with Fehling's or Benedict's reagent, see p. 29.

### Nylander's Test

Bismuth subnitrate in alkaline solution (bismuth hydroxide) is reduced by glucose and other reducing sugars to metallic bismuth on heating. The test is not now used widely, and is open to several fallacies. Thus protein will give a colour change similar to glucose, and must, therefore, be removed from the urine by boiling and filtering before applying the test. Urines rich in indican or containing large quantities of urochrome, uroerythrin or porphyrin may darken when boiled with Nylander's reagent, and yield precipitates similar in appearance to that obtained on testing a urine containing sugar. Apart from these limitations, however, the test is a useful one.

### THE SUBSTANCES WHICH REDUCE CUPRIC SOLUTIONS

In addition to glucose, other reducing sugars may be present in urine, viz., fructose, lactose and pentoses. Maltose and galactose have been reported, but their occurrence in human urine is so rare that they need not be considered. As already mentioned, in concentrated urines a reduction by creatinine and uric acid may occur. Likewise a slight reduction may be caused by glycuronates. In addition to those drugs which have been conjugated with glycuronic acid, there are a few others of which the excretion products may reduce copper solutions, e.g., salicylic acid following the administration of salicylates. Lastly, homogentisic acid, which is excreted in the rare condition alkaptonuria, also reduces cupric solutions.

There are three main tests which are employed in clinical work for the identification of reducing substances, viz., fermentation test, preparation of osazone crystals, and examination with a

polariscope The results of the application of these three tests are given in the following table —

Reducing Substance	Fermenta tion Test	Formation of an Osazone	Rotation of Plane of Polarised Light
Uric acid and creatinine	Neg	No	Nil
Glycuronates (drugs)	Neg	Yes (like glucosazone)	Laevo (free acid dextro)
Salicyluric acid	Neg	No	Nil
Homogentisic acid	Neg	No	Nil
SUGARS	Dextrose (glucose)	Positive	Glucosazone
	Lævulose (fructose)	Positive	Yes (identical with glucosazone)
	Lactose	Neg	Lactosazone
	Pentoses	Neg	Yes (like glucosazone)
			Vary some in- active others dextro

The fermentation test is the most useful in clinical work, because it excludes everything except glucose and fructose. A positive result must signify the presence of one or other of these two sugars. Fructose may be detected by Selwanoff's test (see later). It is not generally realised, perhaps, how limited is the value of the osazone test, as generally carried out in medicine, *i.e.*, preparation and microscopic examination of the crystals. It is not usually a practical proposition to separate, purify and determine the melting point of the osazone. It will be seen from the table above that the successful preparation of crystals broadly speaking, indicates that sugars or glycuronates are present. When typical lactosazone crystals are obtained, there is no mistaking them, but the concentration of lactose in urine is usually small, and it is often not easy to prepare lactosazone crystals at all, about 0.5 per cent of lactose must be present for osazone crystals to be obtained by the ordinary method (White). Moreover when the crystals are few and small it is often impossible to be sure simply from their form, from what sugar they have been derived. Unfortunately it is particularly in such cases, with mere traces of reducing substances, that tests for identification are required. In conclusion, the osazone test is regarded as a useful confirmatory test, but the fermentation test should always be performed simultaneously.

The adsorptive action of charcoal may be used for increasing the sensitivity of the osazone test. Thus Hassan treated normal urines with charcoal to remove interfering substances and obtained osazone crystals from the filtrate. Cole (see his *Practical Physiological Chemistry*) uses charcoal for adsorbing lactose from urine in lactosuria, the charcoal precipitate is separated and

extracted with acetic acid, and the osazone test is performed on the concentrated extract, glucose and pentoses also are adsorbed to some extent, so Cole's technique is not specific for lactose

As a means for identification of small amounts of reducing substances in urine, polariscope examination is of little value. This is not surprising if it be remembered that with the ordinary type of polariscope a 1 per cent solution of glucose only gives a rotation of the order of 1 degree. In clinical work, therefore, this last test is frequently omitted.

### SCHEME FOR IDENTIFICATION OF REDUCING SUBSTANCE

The following is a practical method of investigating urines containing small quantities of reducing substance. As indicated on p 106, it is not required when there is much "sugar" and clinical symptoms of diabetes are present.

Confirm the reported presence of reduction using 5 c.c. of Benedict's reagent and 0.5 c.c. of urine. Blackening of the boiled mixture would at once lead to the suspicion of alkaptonuria, in which event apply the special tests given on p 119.

Next test a portion of the urine with 10 per cent  $\text{FeCl}_3$  solution for salicylates (cf p 231). The excretion of salicylic acid after the administration of aspirin or salicylates is a frequent cause of a slight reduction, so it is wise to consider this possibility straight away. If the ferric chloride test is positive, request that the drug be stopped and test subsequent samples of urine to show that the reduction has ceased.

Then perform the fermentation test. As already noted, *if the fermentation test is positive* the reducing substance must be glucose or fructose. To distinguish between these two, Seliwanoff's test is performed.

#### Seliwanoff's Test for Fructose (Lævulose)

To 5 c.c. of Seliwanoff's reagent (0.05 per cent resorcinol (m dihydroxybenzene) in 33 per cent v/v HCl) add 0.5 c.c. of urine and bring just to the boil. The presence of lævulose is indicated by a red colour. Treat 0.5 c.c. of normal urine similarly for comparison. The test will detect 0.1 per cent of fructose readily, unless much glucose also is present for glucose reduces the sensitivity. Further if the amount of glucose is large it alone may give a positive reaction, this possibility is increased if boiling be prolonged. *Fructose is decomposed by HCl more readily than is glucose to yield a hydroxymethyl furfuraldehyde which condenses with resorcinol to form a red compound.*

*If the fermentation test is negative*, the possible causes of reduction are lactose, pentose, glycuronates, uric acid and creatinine, and slight traces of glucose insufficient to be detected by fermentation (homogentisic acid and salicylic acid have been dealt with). Therefore perform next the osazone test. The possible findings are —

(a) Typical sheaves of phenyl glucosazone (Fig 26, p 114), which considered together with negative results for other reducing substances and the clinical history, are sufficient to diagnose glucose.

(b) Typical "hedgehog" crystals of phenyl lactosazone which show that lactose is present provided that the clinical history is

compatible; but as noted previously (p. 109), most cases of lactosuria do not yield typical crystals (Fig. 26, p. 114.)

(c) Spherical clusters of small acicular crystals (Fig. 26, p. 114) which may be due to lactose, glycuronates or pentoses.<sup>1</sup> In this case the special investigations given below must be carried out.

(d) A negative osazone test, in which case the reduction may be due to traces of lactose or of glycuronates, or to uric acid and creatinine, and again the further investigations are required.

The special investigations referred to under (c) and (d) are as follows:—

If the reduction occurs in the urine of a nursing or pregnant woman, or in the urine of an infant, especially when milk sugar has been added to the feeds, lactose should be suspected; adsorption by charcoal may be tried (see p. 109) in order to obtain lactosazone crystals, but the clinical history, coupled with negative results for other reducing substances, is often the most useful guide.

If drugs conjugated with glycuronic acid (for list of such drugs, see p. 117) are suspected as the cause of the reduction, tests for drugs in urine should be performed (see Chapter XI) and the effect of discontinuing the drug noted. Glycuronates conjugated with putrefactive bodies (see p. 117) are in theory a possible cause of a slight reduction, but in practice the writer has never met with an example. There are no simple and trustworthy tests for glycuronates in urine.

Bial's orcinol test is most commonly employed for the recognition of pentose in urine, it is important, however, that the proportion of urine recommended below be adhered to, otherwise false positive reactions may be obtained with urines containing glycuronates or even with normal urines

### Bial's Test for Pentoses

The reagent is prepared by dissolving 0.4 gm. of orcinol (m-dihydroxy toluene) in 200 c.c. of concentrated A.R. hydrochloric acid (S.G. 1.15 to 1.16) and adding 0.5 c.c. of 10 per cent ferric chloride.

To 5 c.c. of Bial's reagent in a test tube add 0.5 c.c. of urine and bring just to the boil. Allow the mixture to stand for five to twenty minutes. Under these conditions pentoses yield a green colour and, if present in quantity, a bluish green precipitate. Glycuronates will give the same reaction only if the urine and reagent are thoroughly boiled together.

Spectroscopically the green fluid shows a characteristic absorption band between the C and D lines and overlapping the D line (Fig. 48, p. 220). A second band nearer the red end of the spectrum and a fainter band in the green are not of diagnostic significance (Garrod). The green pigment is extracted on shaking with amyl alcohol, and the extract has the same absorption band(s). It is helpful to treat 0.5 c.c. of normal urine similarly for comparison. Bial's test will detect readily 0.1 per cent. of arabinose added to normal urine. If glucose or fructose also is present, it may be removed first by fermentation. Urine containing fructose gives a deep red colour when tested by Bial's method, as might be expected from the similarity of the test to that of Selwanoff, for in both methods the carbohydrate is decomposed by HCl to yield aldehydes of the furfural type, which condense with the phenol (resorcinol, orcinol) to form coloured compounds.

An estimation of pentose may be made in urine by McCance's method, if the nature of the pentose is known.

<sup>1</sup> Pentosazones (Fig. 26, p. 114) after purification have M.P. 156 to 160° C., and nitrogen content of 17.07 per cent.

As an aid to memory, it may be noted that in each of three tests, Benedict's qualitative, Selwanoff's and Bial's, 5 c c of reagent and 0.5 c c of urine are employed.

Lastly, if the above investigations have failed to account for a slight reduction, provided that the urine is concentrated, it may be ascribed to uric acid and creatinine.

It should be remembered that the reduction of cupric solutions may be due to a mixture of substances, *e g*, dextrose and lactose, dextrose and pentose, etc.

For further tests and fuller details as to the separation and identification of the various reducing substances, the reader is referred to larger works.

### Technique of the Fermentation Test

Glucose and fructose are fermented by yeast with the formation of alcohol and carbon dioxide. The test as applied in clinical medicine consists in the demonstration of gas ( $\text{CO}_2$ ) formation and the subsequent testing of the fermented urine with cupric solution to see whether all the reducing substance has been removed. Gas formation is usually obvious in two to four hours if the test is performed at  $37^\circ \text{C}$ . Fermentation is usually complete in twenty-four hours.

Many forms of apparatus have been devised from an inverted test tube to an Einhorn's saccharometer (Fig. 24). A simple and an inexpensive form which has

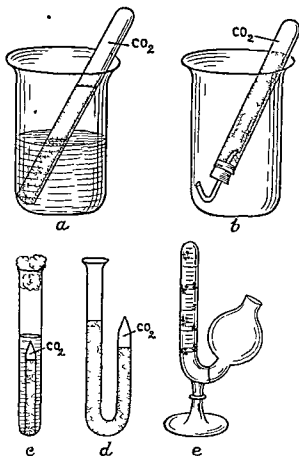


FIG. 24 Fermentation tubes (a to d) and Einhorn's saccharometer (e)

been used in the author's laboratory for several years is shown at (d). A piece of glass tubing is closed at one end and bent into the form of a U tube. The urine and yeast, etc., are mixed in a test tube, and the contents of the tube are transferred to the U tube, which is easily filled by tilting, and so displacing the air which escapes through the open limb.

In order to carry out the fermentation test satisfactorily, several control tubes should be put up. In the first place, the yeast (brewer's, baker's or compressed) should be thoroughly washed with distilled water to remove adherent sugar.

Take a piece of yeast, the size of a large bean, and shake it up thoroughly with distilled water. Filter (e.g., on a Buchner funnel) and wash the yeast on the filter paper again with water to remove all sugar. Boil the urines to kill organisms and to drive off gases in solution, and allow them to cool to room temperature or to 37° C. Take four test tubes, A, B, C and D, and place a portion of the washed yeast, the size of a pea in each. To A add about 10 c.c. of the cooled boiled urine to be tested. To B add about 10 c.c. of cooled boiled normal urine. To C add about 10 c.c. of cooled boiled normal urine together with a large knife point of solid glucose. To D add about 10 c.c. of distilled water. Thoroughly mix the contents of each test tube by shaking set aside for a few minutes to allow air bubbles to escape and then transfer some of the contents to corresponding U tubes, A, B, C and D as illustrated in Fig. 25. Place the four U tubes vertically in some convenient receptacle (e.g., a cigarette tin with sides perforated) in a water bath at 37° C. Usually gas formation will be obvious in two to four hours.

In tube D there will be no gas if the yeast has been washed properly. In tube C there should be a large quantity of gas, this is to show that the yeast is of good quality and is working well. In B there is usually no gas though occasionally a minute bubble may form, presumably owing to the fermentation of the minute amount of sugar which occurs in normal urine. It is not absolutely essential to

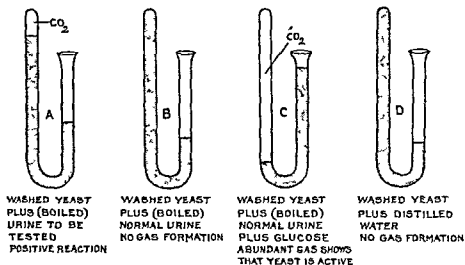


FIG. 25 Fermentation test

put up normal urine as a control in this way, but it is instructive particularly as a comparison in cases in which tube A shows only a small bubble. In tube A there will or will not be gas depending on whether fermentable sugar is present or not (Fig. 25).

At first sight the above procedure may seem unnecessarily complicated but it renders the test much more delicate and conclusive in just those cases where it is most wanted viz., cases in which the concentration of reducing substance is small.

The author has seen students misled by each of the points which the above procedure avoids, viz., evolution of gas by organisms other than the yeast, gas driven out of solution by rise in temperature from that of the room to 37° C., gas formed from the sugar adherent to unwashed yeast, absence of gas owing to inactive yeast, and absence of gas owing to inactivation of yeast by mixture with very hot urine.

Next day, some of the urine in tube A is again tested with Benedict's solution to see whether or no all the reducing substance has disappeared. At the end of the test it is a wise precaution to render the urine in the fermentation tube strongly



alkaline with NaOH, when, of course,  $\text{CO}_2$  will be absorbed. Any unabsorbed gas will be air that has been introduced accidentally when filling the fermentation tube.

### Technique of the Osazone Test

**USING PHENYLHYDRAZINE HYDROCHLORIDE** Take about 10 c.c. of urine in a test tube, and make acid with acetic acid. Then add as much phenylhydrazine hydrochloride as will lie on a sixpenny piece and twice that bulk of sodium acetate. Mix thoroughly, heat till solution has occurred, and filter. Place the tube containing

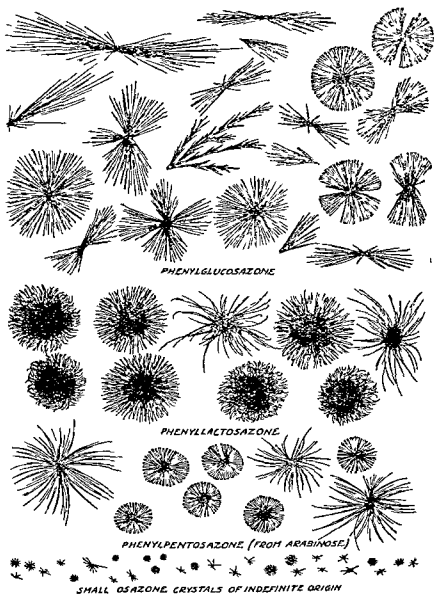


FIG 26 Osazone crystals

the filtrate in a boiling water bath for half to one hour. Then remove the flame and allow the filtrate to cool down slowly in the water bath to room temperature. Mount any precipitate which forms and examine under the microscope.

**USING PHENYLHYDRAZINE (free base) (Neumann)** To approximately 10 c.c. (2 in. column in a test tube) of urine add 2 c.c. of saturated sodium acetate in 50 per cent acetic acid, and 5 or 6 drops of phenylhydrazine. Mix and place in a boiling

water bath. There is no need to filter. Frequently osazone crystals will appear in ten or fifteen minutes. If not, leave for half an hour, and then turn out the flame, but do not remove the tube. Mount and examine any crystals under the microscope.

## CHEMICAL TESTS IN INTERMITTENT "GLYCOSURIA" (i.e., WHEN REDUCING SUBSTANCES APPEAR INTERMITTENTLY)

The occurrence of traces of reducing substances in the urine is one of the bugbears of life insurance work. Similar problems occur in other routine urinary examinations, e.g., before operation, etc.

The first thing to do is to determine the nature of the reducing substance as described in previous sections. If the reducing substance is not glucose it can be ignored from a purely practical point of view, if the clinical condition is sound. If the reducing substance is glucose, then a blood sugar estimation should be performed. The interpretation of the blood sugar finding is dealt with in the next chapter, but if the glycaemia is 200 mgm per 100 c.c. or over, the glycosuria is almost always pathological. If the blood sugar is less than 200 mgm, or if there is any doubt after the isolated blood sugar test, a blood sugar curve should be made in order to decide the significance of the glycosuria (Chapter VII).

If ketonuria accompanies the glycosuria, diabetes mellitus should be suspected (and carefully excluded), but it should be remembered that ketonuria (Chapter IX) may occur in many conditions apart from diabetes. The combination of glycosuria with ketonuria is by no means diagnostic of diabetes.

## THE CLINICAL SIGNIFICANCE OF THE VARIOUS REDUCING SUBSTANCES

### Glucose or Dextrose

It is a good rule when abnormal amounts of dextrose in the urine have been demonstrated, to regard the case as one of diabetes mellitus unless some definite cause for the glycosuria can be found. The significance of glycosuria will be discussed more fully in the next chapter in connection with the interpretation of blood sugar curves. Here it is only necessary to note that there are many causes of glycosuria besides diabetes. Of these the most important clinically are (1) renal glycosuria which is of two types (a) with and (b) without a demonstrable kidney lesion, (2) lag glycosuria, (3) glycosuria due to disturbances of the ductless glands, (4) glycosuria associated with infectious and septic conditions, (5) glycosuria associated with increased intracranial pressure (meningitis, cerebral hæmorrhage, fractured skull, intracranial tumours, etc.). For a full discussion of glycosuria the reader is referred to Allen's book. It is rare to find glycosuria in liver disease uncomplicated by pancreatic disease. Opinion is divided as to whether or no a true physiological "alimentary glycosuria" exists at all. According to some authorities it is impossible to cause glycosuria in healthy individuals however large the dose of glucose. Provided that renal glycosuria and lag glycosuria have been excluded

by blood-sugar curves (see next chapter), in actual practice it must be seldom, if ever, that the alimentary glycosuria is not pathological

### Fructose or Lævulose

Fructosuria is a relatively uncommon condition. Lævulose is sometimes excreted together with glucose in diabetes mellitus, but in diabetes it is not likely to be detected unless specially looked for, and it has no special significance in this condition. Alimentary fructosuria is occasionally encountered, this is generally due to ingestion of fructose as such, but a few "spontaneous" cases have been reported which are regarded as having been caused by the eating of food containing fructose, *eg*, certain fruits. In the laboratory fructosuria is commonly met with in the lævulose test of liver efficiency (see Chapter XII). The renal threshold is certainly much lower for fructose than for dextrose. Thus the lævulose test, when performed on healthy adults, often leads to excretion of the sugar even after doses as small as 50 gm.

The occurrence of fructosuria suggests the necessity of an inquiry into the condition of the liver, but fructosuria itself calls for no treatment. In cases of alimentary fructosuria without hepatic lesion the blood sugar lies within normal limits. Reference to the blood sugar in the lævulose test of liver efficiency is made later (see Chapter XII). In diabetes, fructosuria is usually associated with hyperglycæmia and dextrosuria.

### Lactose

Lactosuria is common in nursing mothers, and is particularly apt to be found in mothers who recently have had to cease suckling. It has often been stated that lactosuria is not uncommon in the last few weeks of pregnancy, but Winter found that the urines of only two out of twenty seven mothers at this time contained a reducing substance which in each case was glucose, further investigation is required. Lactose may appear in the urine of patients who have been on a milk diet for a long time. Lactosuria has also been reported in infants, particularly when lactose has been added to the feeds. It may occur in breast fed infants suffering from gastro enteritis. An alimentary lactosuria may occur in healthy individuals after ingestion of lactose. The actual limit of assimilation in adults probably varies considerably in different individuals. At any rate, the limits given by different workers vary from 30 to 120 gm. Folin and Berglund state that lactose as such is present in the urine after the ingestion of lactose unless the amount taken is less than 30 gm.

Pure lactosuria does not require any treatment. Its importance lies in the differentiation from true glycosuria. In cases of pure lactosuria the blood sugar lies within normal limits.

### Pentoses

An alimentary pentosuria (pentosanuria) is occasionally encountered in the "fruit season". An inquiry should be made

into the diet, particularly as regards the ingestion of large quantities of cherries, damsons, plums, or fruit juice. The kidneys excrete all the pentoses which reach them. The ingestion of even  $\frac{1}{2}$  gm of pure arabinose may cause pentosuria. A few diabetics excrete pentoses together with dextrose. Otherwise pentosuria is practically confined to rare individuals suffering from an inborn error of metabolism. The Jewish race appears to be particularly prone to the condition, in which the amount of pentose excreted usually lies between 1 and 7 gm in twenty four hours.

The occurrence of pentose in the urine does not call for any treatment, though, of course, associated conditions (*e.g.*, diabetes) may need attention. Very few estimations of the sugar in the blood have been made in pentosuria, but in the reported cases the blood sugar has been normal except in those instances in which pentosuria is an accompaniment of diabetes (*cf.* Rabinowitch).

### Glycuronates

Glycuronic acid may be excreted in the urine in combination either with drugs or with products of intestinal putrefaction. The formation of these compound glycuronates is a protective mechanism, and the seat of their formation is the liver. In this way the liver renders such substances innocuous before passing them on to the general circulation from which they are finally excreted by the kidneys in the urine. The chief drugs thus conjugated are antifebrin (acetanilide), antipyrine (phenazone), pyramidone (amidopyrin), camphor, chloroform, chloral, morphine, menthol, naphthol, phenol, thymol oil of turpentine, etc. The chief putrefactive bodies thus conjugated are indoxyl, skatoxyl, phenol, paracresol, etc. As already mentioned, when drugs are suspected as the cause of glycuronate formation, the effect of discontinuing the drug should be observed. If excessive intestinal putrefaction is suspected, the effect of a purge should be tried. Putrefactive bodies may be excreted as glycuronates, or as ethereal sulphates, or in both ways but indicanuria is not necessarily accompanied by an excessive excretion of glycuronates. When the urine contains traces of a reducing substance a positive test for indican should not be regarded as evidence in favour of the reduction being due to glycuronates.

### Reduction following Administration of Drugs other than those Conjugated with Glycuronic Acid

Salicylates have already been mentioned as being excreted (in part) as salicyluric acid. This is an example of kidney synthesis, glycine (amino acetic acid) being combined with salicylic acid (o hydroxy benzoic acid) to yield salicyluric acid (o hydroxy benzoyl glycine, or o hydroxy hippuric acid) (Chapter XI). The latter reduces cupric solutions if present in more than traces.

Owing to the great frequency of administration of salicylates and their derivatives—particularly aspirin (acetyl salicylic acid)—they are a very common cause of slight reductions of cupric solutions,

so much so that, as noted previously, it is a good plan to test for salicylates by adding ferric chloride solution (*cf* Chapter XI) to all urines which have been found to contain traces of "sugar"

### Alkaptonuria

This is a very rare condition, and is due to an inborn error of metabolism whereby tyrosine and phenylalanine are incompletely broken down, with the result that homogentisic acid (paradihydroxy phenyl acetic acid) is excreted in the urine. The homogentisic acid is formed both endogenously and exogenously, its daily output commonly amounts to 4 or 5 gm.

The fresh urine is of normal colour. On standing it turns brown in a few hours, and may eventually become black. This darkening is due to the conversion of homogentisic acid by atmospheric oxygen into brown or black oxidation products the chemistry of which is not known, the final oxidation product behaves in many ways like melanin, and may be termed "melanin like" until such time as it has been more fully studied. The darkening of the urine is much more rapid with an alkaline than with an acid reaction, but darkening in air does occur slowly even though the reaction be kept acid.

When the urine is allowed to stand without the addition of a preservative, it becomes alkaline in a few hours owing to the conversion of urea into ammonium carbonate by bacterial action and it turns brown. If the sample is not disturbed it darkens from the surface downwards. If alkali (ammonia, caustic soda or potash) is added in excess, the fresh urine darkens in a few seconds, particularly if well shaken.

If toluene is added as a preservative, both the admission of air and the multiplication of bacteria are retarded, but not stopped, so that the darkening is slower. Darkening may be prevented for weeks or even months by adding 5 c.c. of sulphurous acid (water saturated with  $\text{SO}_2$ ) to each 100 c.c. of urine, but unfortunately this preservative interferes with subsequent testing. The free  $\text{SO}_2$  may be removed by adding porcelain chips and boiling vigorously for a few minutes, but some of the added  $\text{SO}_2$  invariably combines with urinary bases to yield soluble sulphites which cannot be so simply removed. Moreover, even after removal of free  $\text{SO}_2$ , the reaction of the urine is very acid, neutralisation with  $\text{NaOH}$ , however, fails to make the ferric chloride test satisfactory, although it enables the reduction of salts of copper and silver to be demonstrated properly.

Owing to the above mentioned oxidation of homogentisic acid, linen soiled with alkapton urine becomes stained reddish brown or brown. This may be the initial observation that leads clinically to the detection of the condition, but probably the behaviour of the urine when tested with cupric solutions more often leads to the diagnosis.

When alkapton urine is boiled with Benedict's qualitative reagent, the mixture assumes a strikingly greenish brown or greenish

black hue, the precipitate at first appears dirty brown in colour, but on standing the usual yellow colour of cuprous oxide is evident. With Fehling's solution a similar appearance is obtained, but the heated mixture is black, the deeper pigmentation being due to the use of a larger proportion of urine in Fehling's than in Benedict's test, the precipitate at first appears greyish black, but later separates and is red.

If the alkaptan urine is layered upon the Benedict's or Fehling's solution, a brown ring forms at room temperature at the junction of the two fluids, owing to the action of the alkali of the cupric solution.

Alkaptan urine is neither dextro rotatory nor lævo rotatory, and is not fermented by yeast.

It reduces ammoniacal silver solution in the cold. This may easily be shown by adding about 0.5 c.c. of the urine to about 5 c.c. of 3 per cent silver nitrate, and mixing, on adding a few drops of 10 per cent v/v ammonia solution, the mixture turns black.

It is not so widely known that alkaptan urine will reduce silver nitrate in the cold in the absence of ammonia, although the observation is an old one (*cf* Smith). If about 0.5 c.c. of the urine is mixed with about 5 c.c. of 3 per cent silver nitrate, there is an immediate white precipitate of silver chloride, and then the mixture, in a few seconds to a minute or two, turns grey, grey black with a blue sheen (due to colloidal silver) and finally black. In the writer's opinion this method is to be preferred to the previous one for clinical purposes, the presence of ammonia makes the test almost too sensitive, because normal urine will sometimes give a dirty brown, though never a black colour, in the absence of ammonia there is no darkening at all with normal urine.

The most characteristic test in alkaptanuria is the colour reaction with ferric chloride. If to about 5 c.c. of the urine in a test tube, ferric chloride solution (1 to 10 per cent) be added drop by drop, there is, in addition to the precipitate of ferric phosphate, a transient green or blue colour. After the addition of a few drops, oxidation is complete, and further ferric chloride produces no colour change. For this reason it is safer to use the more dilute solution. The reaction may also be demonstrated well by adding drop by drop 1 per cent ferric chloride to 5 or 6 drops of the urine on a porcelain tile with shallow cavities. In most recent descriptions of the test the colour is described as "deep blue," but it is quite often green and not blue, an observation which again is an old one (*cf* Smith), the chemistry of this colour reaction is not known.

Melanogen, which is excreted in some cases of visceral melanotic sarcoma, is oxidised on standing to melanin, so that the urine similarly darkens from the surface downwards. Urines containing melanogen, however, do not usually reduce cupric solutions, and are easily differentiated because they do not give a transient green or blue colour with ferric chloride. A table summarising the reactions in alkaptanuria and melanogenuria respectively, is given in Chapter XI.

In later years, usually after the age of forty, the cartilages of alkaptonurics become blackened (presumably by the melanin like oxidation products of homogentisic acid), giving a blue tint to the hollows of the ears and brown markings on the conjunctivæ. The blackening of the cartilages and ligaments is known as "*ochronosis*". It should be noted that *ochronosis* may also follow in man from the prolonged use of carbolic acid. The term "*ochronosis of cattle*" has unfortunately been applied to a condition in which the bones are stained dark brown by porphyrins.

## QUANTITATIVE ESTIMATION OF SUGAR IN URINE

There are literally dozens of methods for the estimation of sugar in urine, but for routine use there is none simpler than Benedict's method. Some writers prefer a polarimetric estimation, but in my experience in clinical work it is no quicker than Benedict's method, because of the time spent in removing pigments and clearing the urine before examination in the polarimeter. In fact, owing to the frequent occurrence of levorotatory substances (*e.g.*,  $\beta$  hydroxybutyric acid) the polarimetric method is often inaccurate.

From a purely clinical point of view accurate estimations of the glucose excreted are a waste of time unless the intake of food is accurately measured. The clinician can obtain all the information he requires from a rough test with Fehling's solution, in that if the diet is uncontrolled, all he wants to know is whether the urine contains small, moderate or large amounts of sugar. This rough test with Fehling's solution will, therefore, be described first.

It cannot be emphasised too strongly that it is necessary to estimate the *total* excretion of sugar in the twenty four hours if the effect of any particular line of treatment is being watched. This means a complete collection and measurement of all the urine passed in the twenty four hours—a condition not always easy to fulfil in practice. A comparison of the *concentrations* of sugar in isolated specimens may be very misleading, owing to the influence of fluid intake etc. Thus in the fasting treatment of diabetes it was not uncommon for the concentration of sugar to *increase* at first, though the total excretion per diem was decreased. Marked polyuria was often replaced by oliguria, thus readily accounting for the observation.

### Rough Test with Fehling's Solution

Place 1 in. of urine in one test tube and 1 in. of Fehling's solution in another. Heat the two tubes over the same flame simultaneously, and when boiling, mix by pouring from one tube to the other and back again repeatedly as quickly as possible. If the Fehling's solution turns yellow at the first mixing there is, very roughly, 7 per cent. or more of sugar present. If the yellow colour does not appear until the second mixing, there is roughly 4 to 5 per cent. of sugar. If there is a reduction on the third mixing there is

approximately 2 per cent, and if further pouring to and fro can be made before the yellow colour appears there is less than 2 per cent of sugar. In other words, the test shows whether large, moderate, small, or very small concentrations of sugar are present.

With practice it is easy by this means to judge the percentage of sugar sufficiently closely to enable one to calculate the preliminary dilution which is often necessary in Benedict's quantitative test.

### Quantitative Estimation of Sugar in Urine by Benedict's Method

**Preliminary.** Benedict's quantitative solution (see Appendix) is made up so that 25 c.c. are reduced by 0.05 gm. of dextrose. It is desirable to dilute the urine so that 10 c.c. of the diluted urine contain about 0.05 gm. of dextrose, i.e., so that the concentration of sugar is approximately  $\frac{1}{2}$  per cent. For this reason perform the rough test with Fehling's solution as described above. Make the dilution in accordance with the result, thus —

No. of Mixings	Approximate Percentage of Glucose	Dilut on
1	7 or more	1 in 15 to 1 in 20
2	4 to 5	1 in 10
3	About 2	1 in 4
4 or more	Less than 2	Use neat urine

The dilution must be made accurately, e.g., measure 10 c.c. of the urine with a pipette into a 100 c.c. volumetric flask, and fill up to the mark with distilled water to obtain a dilution of 1 in 10.

**Principle.** Glucose and other reducing sugars reduce cupric sulphate in boiling alkaline solution to cuprous oxide or hydroxide, which is red or yellow. It is difficult to see when the reduction of the copper solution is just completed as shown by the disappearance of the final green tinge against a yellow background. Benedict, therefore, introduced sulphocyanide and ferrocyanide into his reagent, the former gives a white precipitate with cuprous hydroxide, thereby rendering the end point more definite the latter also tends to prevent the deposition of cuprous oxide.

**Method of Estimation.** Fit a 100 c.c. round bottomed flask on an asbestos wire gauze supported on the ring of a retort stand, the neck of the flask being loosely held in a clamp (Fig. 27). Place in the flask 3 to 4 gm. (roughly measured in a test tube marked for the purpose) of anhydrous sodium carbonate, a few pieces of porous porcelain and 25 c.c. of Benedict's quantitative solution. Bring to the boil, and run in the (diluted) urine from a burette held in the hand, 10 to 20 drops at a time, boiling between each addition. As soon as a bulky white precipitate is seen run in the urine more and more slowly (5 or 6 drops, and finally 1 or 2 drops at a time), with constant boiling until the last trace of blue or green has disappeared. If too much urine be added the solution will become



yellow owing to caramelisation of the excess sugar by the alkali in the reagent (After the estimation is finished correctly, the titrated

mixture, on standing, will turn green again owing to oxidation by air) Note the volume of (diluted) urine used

If the mixture tends to bump before the titration is complete, remove the flame, boil approximately 10 c c of water in a test tube and pour into the flask. Then bring to the boil and continue the titration. Place the tip of the burette over the mouth of the flask only whilst actually running in the urine. If the burette tip is left permanently in the mouth of the flask, urine will be displaced by the steam issuing from the flask, thereby introducing an error.

**Example of Calculation** The urine was diluted 1 in 4 12.6 c c of the diluted urine were required to reduce completely 25 c c of Benedict's solution

25 c c of Benedict's solution are reduced by 0.05 gm of dextrose

Therefore 12.6 c c

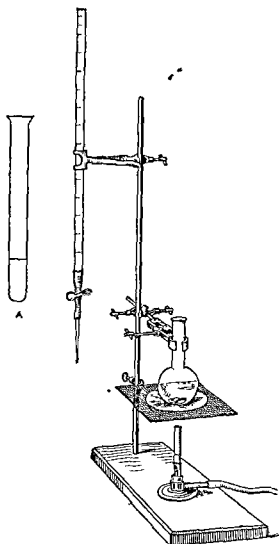
FIG 27 Apparatus for estimation of sugar in urine by Benedict's method. A Marked test tube for 3 to 4 gm of anhydrous sodium carbonate

of diluted urine contain 0.05 gm of dextrose

Therefore  $\frac{12.6}{4} = 3.15$  c c of original urine contain 0.05 gm of dextrose

Therefore 1 c c of original urine contains  $\frac{0.05}{3.15}$  gm of dextrose

Therefore 100 c c of original urine contain  $\frac{0.05 \times 100}{3.15} = \frac{5}{3.15}$   
 $= 1.59$  gm of dextrose



Concentration of dextrose = 1.59 per cent

Volume of urine in 24 hours = 1,700 c c

Therefore quantity of dextrose excreted in 24 hours

$$= 1.59 \times \frac{1,700}{100} = 27 \text{ gm}$$

## REDUCING SUBSTANCES IN NORMAL URINE

In clinical work as a general rule no information of value is to be obtained by estimating the reducing substances in urines which do not reduce Benedict's qualitative solution. It is important to realise, however, that normal urine does contain traces of reducing substances. Occasionally, moreover, a patient's urine is so concentrated that a slight reduction of Benedict's qualitative solution does occur (see p. 112).

The nature of the reducing substances in normal urine was for a long time a disputed point, particularly with regard to whether glucose is or is not present. The work of Harding and Selby, West and Steiner leaves little room for doubt that glucose is present, both after a night's fast and after meals.

In health a total of about 1 gm. of reducing substance is excreted in twenty-four hours. About  $\frac{1}{2}$  gm. is fermentable, and is probably mainly glucose and fructose. About  $\frac{1}{2}$  gm. is non fermentable and includes pentoses and glycuronates.

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## CHAPTER VII

### THE INTERPRETATION OF BLOOD-SUGAR AND BLOOD-SUGAR CURVES

**Books.** Cameron and Gilmour's *The Biochemistry of Medicine*  
MacLean's *Modern Methods in the Diagnosis and Treatment of Glycosuria and Diabetes*

De Wesselow's *Chemistry of the Blood in Clinical Medicine*

Joslin's *Treatment of Diabetes Mellitus*

Graham's *Pathology and Treatment of Diabetes Mellitus*

H Gray, "Blood Sugar Standards Normal and Diabetic Persons"  
*Arch Int Med*, 1923, 31, 241 "In Conditions neither Normal nor Diabetic," 259

IN order to obtain data for a blood sugar curve it is necessary to estimate the concentration of sugar in the blood (see end of this chapter) and in the urine (Chapter VI) repeatedly at set intervals, after a meal or after a dose of sugar. The figures so obtained are presented in the form of a curve, and the study of such curves is often of value in mild glycosurics, in controlling the treatment of diabetes, and also for appraising the meaning of isolated determinations at known intervals after food. The findings in normal individuals, the factors influencing the curves, and the variations caused by various diseases will be reviewed in order. Except where otherwise stated, all the sugar results refer to capillary blood. It is important to note this, as will be explained later. It must also be emphasised that the blood sugar varies *slightly* with the method used. Most of the reducing substance in blood is glucose, but there is also a small amount of non glucose reducing substance, and it is owing to the inclusion of varying proportions of this in the total reduction that the results differ. The subject is considered later in this chapter.

#### THE NORMAL CURVE

As examples of the results in normal man, (a) the curve after a standard dose (50 gm.) of dextrose, taken fasting, and (b) the curve after a mixed meal of average size will be discussed.

(a) A typical curve for an adult of, say, twenty five to forty years, after 50 gm. of dextrose, is given in Fig 28, and the lower and upper normal limits in Fig 29. The blood sugar is usually estimated before, and at half hourly intervals after this dose taken fasting, up to a period of two hours and sometimes for longer. The urine is obtained for the same periods, if possible. Frequently one has to be satisfied with hourly samples of urine, and sometimes a specimen before and another at the end of the test is all that can be

secured. The urine tests should never be omitted, they are just as important as the blood tests. In the charts the percentage of sugar is plotted vertically and the time horizontally.

Time in hours	0	$\frac{1}{2}$	1	$1\frac{1}{2}$	2
Blood sugar, mgm per 100 c c	100	160	120	80	100
Glycosuria	Nil throughout				

Fifty grammes are selected as the test dose because it is sufficiently large to give the maximum response in a normal individual. Indeed, amounts greater than 25 to 30 gm all give very similar curves, except that as the quantity reaches the order

TYPICAL BLOOD SUGAR CURVE  
AFTER 50 gm OF DEXTROSE, D  
TAKEN FASTING IN A NORMAL ADULT

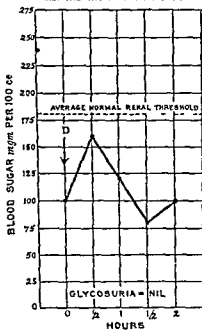


FIG. 28

UPPER AND LOWER NORMAL LIMITS OF  
BLOOD SUGAR AFTER 50 g DEXTROSE

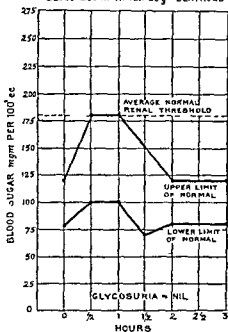


FIG. 29

of 100 gm or more the curve becomes slightly prolonged to the right. In order to calculate the dose for younger individuals it should be noted that 50 gm in an adult corresponds to approximately  $\frac{3}{4}$  gm per kgm, or  $\frac{1}{2}$  gm per lb of body weight. In America it is a common practice to use larger doses of sugar, e.g.,  $1\frac{1}{2}$  gm per kgm.

The preparation for the test is as follows. The patient is told to eat and drink nothing overnight. First thing in the morning, i.e., three hours or more before the test, a cup of tea may be permitted, but no carbohydrate should be allowed. Sometimes, however, it is more convenient to allow a meal some three to four hours before the test. The stricter preparation makes the conditions of the test more standard, but the meal helps to fill the carbohydrate stores which have been partially depleted by the

night's fast in which case the subsequent dose of sugar is more likely to reveal slight deficiencies in the carbohydrate storage mechanism

The 50 gm of dextrose are dissolved in 100 to 250 c c of water. The solution may be flavoured, if desired, with the juice of one lemon. There appears to be no rigid convention in regard to the quantity of water used for making the solution of dextrose, though it is of some importance. With 100 c c the solution is very hypertonic and sometimes causes nausea or vomiting or may delay the absorption of glucose into the blood, moreover, it is more difficult after 100 than after 250 c c of fluid to obtain half hourly samples of urine. An objection to 250 c c is that the patient may find the volume too large. The writer's practice is to give the 50 gm in 150 c c and to follow this by 100 c c of water to remove the sweet taste, refusal to drink all of the last 100 c c is then of less moment.

The points to notice in the normal curve are (i) The fasting level. This normally lies between 80 and 120 mgm per 100 c c, a convenient average figure to memorise being 100 mgm. (ii) The highest point of the curve and the time at which it occurs. Normally this peak is not higher than 170 to 190 mgm, and it occurs within the first hour. (iii) The time taken to return to a normal level of 120 to 80 mgm per 100 c c. Usually this interval is one and a half to two hours. (iv) The absence of glycosuria.

The next points of importance are the variations which may occur in a normal individual. First, there is the influence of age. In infancy the fasting level is frequently as low as 60 to 80, the peak of the curve lies at 100 to 140, and the blood sugar generally returns to its original level in one and a half hours. The level of a child's blood sugar curve approaches that of an adult at the age of about ten years. In old age the general level of the curve tends to rise. Thus the fasting level commonly lies between 100 and 140 mgm per 100 c c, the peak between 180 and 220, and the duration two to three hours. Secondly, it has been shown by Graham and others that the blood sugar curve tends to rise slightly and to be a little prolonged by fatigue. A holiday will often send it back to the original level. Thirdly, worry, mild infections such as a cold in the head etc, probably all have a slight effect on the blood sugar curve similar to that observed by Graham above, though it is difficult to prove this. The effect of emotion is another possible factor. One not infrequently notes in a new patient that the blood sugar on the first visit is slightly higher than the blood sugar on subsequent visits (the estimations being made under similar conditions). Usually, however, it is only in the first out of the five sugar determinations made to provide the curve that this rise is observed. In the writer's experience it is exceptional for emotion to affect the blood sugar significantly.

It has been claimed (Lundberg) that smoking causes a transient slight rise of blood sugar, but in our experience it has no significant influence.

(b) The curves obtained after mixed meals vary considerably, as will be appreciated when the factors influencing such curves are discussed, but the following will give an idea of a typically normal finding (Fig 30) —

Time in hours	0	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$	3
Blood-sugar, mgm per 100 c c	100	160	130	150	140	110	100
Glycosuria	Nil throughout						

It will be noted that the fasting level falls within the normal 80 to 120, and that the highest point never reaches the normal maximum of 180 mgm per 100 c c. The difference from the standard glucose curve is shown by the dip and by the prolongation of the above curve. Normally the blood sugar comes down to the fasting level within three hours after a mixed meal.

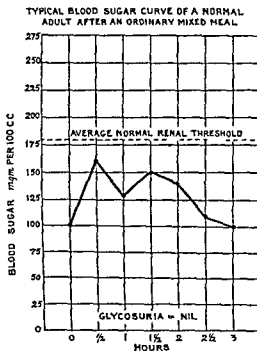


FIG 30

It behaves similarly after each meal containing carbohydrate, so that during the day it oscillates, but at night it remains steady (unless food is taken). In most healthy adults the variation during the day is from 80 to 180 mgm per 100 c c (*cf* p 167), the higher level being approached from one to two hours after each mixed meal, and the lower level being approached from some three hours after each meal until the beginning of the subsequent meal. There are, however, so many factors which influence the blood sugar (*cf* next section) that the rise after each meal is not necessarily proportional to the carbohydrate in that meal. Several investigators (*e.g.*, Shapland) have noted that in some healthy individuals the

blood sugar at about 4 p m is unexpectedly high after quite small amounts of carbohydrate, Harding and Selby have found that 50 gm of dextrose commonly produce a higher curve at 4 p m than first thing in the morning, and may cause "afternoon glycosuria" Again, some individuals may show the usual rise in blood sugar after the first meal (breakfast), but little or none after the second meal (mid day to 1 p m) There is still room for further study of the physiological variations in blood sugar, it is probable that other sub groups could be defined in addition to those already recognised as renal glycosuria" (p 133) and "lag glycosuria" (p 134)

The normal types of curves (Figs 28, 29 and 30) are found in healthy individuals, in an enormous group of pathological states where the carbohydrate metabolism is not affected, and in conditions where the carbohydrate tolerance is "increased" (cf p 131), *e g*, myxœdema dystrophia adiposo genitalis and Addison's disease Furthermore, in diabetes a curve after a restricted meal will frequently be within normal limits, though, of course a curve after glucose would not

### THE FACTORS INFLUENCING BLOOD-SUGAR CURVES

At this point it is convenient to discuss the various factors influencing the form of a blood sugar curve On considering what may happen to food taken by the mouth, it is surprising that the shape of a blood sugar curve is as constant as it is

The blood sugar content of the peripheral blood at any given moment after a meal will depend on (a) the type of food, and the quantity thereof, (b) the rate of digestion, and the rate of absorption of glucose from the small intestine, (c) the glycogenic function of the liver and muscles, (d) the action of the nervous system and of other organs on the glycogen stores, (e) the threshold of the kidneys for glucose, (f) the amount of exercise and the demands of the tissues, (g) the action of the ductless glands viz —

Pancreatic islets	versus	Thyroid
(Insulin)		Pituitary
		Adrenals

and (h) the previous diet The influence of age has already been discussed (p 126)

(a) and (b) Type and Quantity of Food Rate of Digestion and Absorption The effect of pure carbohydrate has been seen in the curve after 50 gm of dextrose Other sugars and rapidly digested starches given in corresponding doses (*e g*, 100 gm of white bread, 240 gm of boiled potatoes, 70 gm of oatmeal as porridge) give very similar curves if taken unmixed with other foods This is not true however, if mixed with much fat, for instance (Jacobsen), so that the passage through the stomach is delayed Thus if the bread is soaked heavily in dripping, or the porridge smothered in

cream, the resulting blood sugar curve may be slightly more prolonged and not so raised. Starches enclosed in cellulose, which is not rapidly digested (*e.g.*, in green vegetables), give a curve which is less high, but more prolonged than the curve after the same weight of less protected starch (*e.g.*, bread).

It is interesting to note that 50 gm of *lævulose* have little or no effect on the blood sugar curve of normal man, the curve approximates to a straight horizontal line. It has been assumed that this is due to the action of the liver in storing *lævulose* as rapidly as it is presented to it, and a test for liver efficiency has been devised on this assumption (see Chapter XII).

Neither protein nor fat has any immediate effect on the blood sugar. Protein on digestion gives rise to amino acids, a large proportion of which is deaminised by the liver, and the fatty acid residues can afterwards yield carbohydrate. It is reckoned that each gramme of protein yields the equivalent of just over  $\frac{1}{2}$  gm of glucose (0.58 gm). Similarly in metabolism each gramme of fat yields the equivalent of 0.1 gm of glucose (from glycerol). But the katabolism of protein and of fat is so slow that the ingestion of either without carbohydrate has no immediate effect on the blood sugar curve as studied over a period of two to three hours. In the case of a mixed meal, however, protein and fat may have a marked indirect effect on the rate of digestion of carbohydrate, and hence on the rate of absorption of dextrose, as has already been exemplified in the case of porridge with cream, etc.

CAPILLARY (BROKEN LINE) AND VENOUS (SOLID LINE) BLOOD SUGAR CURVES IN A NORMAL ADULT AFTER 50 gms DEXTROSE TAKEN INSTEAD OF LUNCH

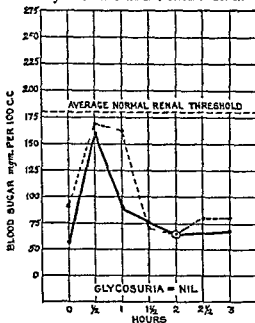


FIG 31

CAPILLARY (BROKEN LINE) AND VENOUS (SOLID LINE) BLOOD SUGAR CURVES AFTER A MIXED LUNCH OF 57 gms OF CARBOHYDRATE 32 gms OF PROTEIN & 43 gms OF FAT IN A NORMAL ADULT

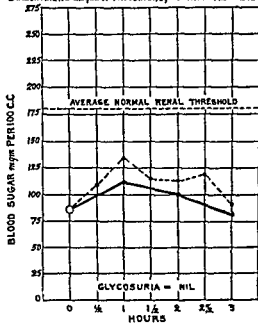


FIG 32



(c) and (d) **Glycogenic Function Action of Nervous System**  
 If the liver is severely damaged the sugar content of the peripheral blood may rise higher than normally after food. This rise is but seldom significant, however, owing to the great reserve power of the liver. In clinical work glucose curves are of little value in liver disease, partly on this account, and partly because of the difficulty of excluding coincident pancreatic disease. The muscles take up glucose from the arterial blood, so that after glucose, and to a lesser extent after an ordinary meal, the venous blood sugar may be definitely lower than the capillary (arterial) blood sugar (Figs 31 and 32). After a night's fast, the normal capillary and venous blood sugars are approximately the same. When recording the results of isolated analyses it is important to note the interval since the last meal, and to state whether the blood is venous or capillary. In diabetes mellitus the difference between the capillary (arterial) and venous blood sugars is often less or absent following carbohydrate meals. Insulin restores the arterio venous difference to normal. In the fasting diabetic the venous may be even slightly higher than the capillary blood sugar (Rabinowitch).

Increased intracranial pressure may cause hyperglycæmia and glycosuria. Thus in cerebral hæmorrhage, tuberculous meningitis, head injuries (cf Davidson and Allen), etc., there would be a raised and prolonged blood sugar curve. Sufficient data for a curve are rarely obtained, but isolated tests often show definite hyperglycæmia.

(e) **Renal Threshold** Normally the kidney does not allow sugar to pass into the urine till the concentration in the blood exceeds about 180 mgm per 100 c c, i.e., the average normal renal threshold for dextrose is 180. The threshold is not an absolutely fixed point, but usually lies between 170 and 190 mgm. It may, however, be lowered as in renal glycosuria, or by kidney disease or by certain kidney poisons, e.g. phloridzin. Conversely, it may be raised. Thus a slight raising of the threshold is common in old age. Gross kidney disease may cause increase of sugar in the blood and cerebrospinal fluid, there being no coincident glycosuria. (It has been shown that the increase in blood 'sugar' is due to glucose.) This condition was observed in two cases of uræmia. The first had 232 mgm of sugar and 570 mgm of urea per 100 c c of cerebrospinal fluid. The second had 211 mgm of sugar and 363 mgm of urea per 100 c c of blood. In neither was there glycosuria. In a sense these are somewhat special cases because it may be said that sugar was not excreted simply because there was little sound kidney tissue to excrete it. The best examples of raised thresholds occur in diabetes mellitus. In this disease the threshold may be raised as a protective mechanism or because the power of the renal cells to excrete glucose becomes impaired after long continued bombardment with excess of sugar.

(f) **Exercise** In normal persons, on an unrestricted diet, moderate exercise slightly elevates the blood sugar, while severe exercise lowers it, but in both cases the sugar tolerance is increased (Allen, Stillman and Fitz). That is to say the blood sugar curve

after a standard dose of dextrose is set at a lower level if the curve is performed whilst the individual is taking exercise, than if the curve is made on the same individual at rest. The same is true in diabetes of mild or moderate grades but is not true of very severe diabetics (without insulin), who tend to become dangerously exhausted by exercise. The beneficial action of exercise on diabetics under insulin control is very marked and the blood sugar may be very extensively lowered thereby (*cf* Lawrence, 1926).

(g) **Ductless Glands** The action of the four most important ductless glands has been indicated schematically above. The action of the internal secretion of the pancreas and of exogenous insulin, is well known. It is probable that the height of the blood sugar is itself the stimulus to the output of endogenous insulin. At any rate, this provides a good working hypothesis on which to base treatment. Insulin possibly enables the tissue cells to "catch hold" of glucose so to speak, preparatory to burning it or to storing it as glycogen. It is, therefore one of the most important factors influencing the blood sugar curve. Its action, of course, is to lower the blood sugar and conversely blood sugar estimations are essential to control the treatment of diabetes by insulin (Chapter VIII).

A few cases have been reported in which hypoglycæmia is due to hyperfunction of the pancreatic islets ('hyperinsulinism') at operation or post mortem either an adenoma or a carcinoma of the islet tissue has been found. The neoplasm may be localised, in which case it can be removed surgically, but it is more often diffused throughout the gland. The symptoms are those of a persistent overdose of insulin and the blood sugar is low except when suitably relieved by carbohydrates. Whipple and Frantz review the published examples (see also Gammon and Tenery).

It has also been suggested (Cambridge, Harris) that certain cases presenting symptoms of hunger, weakness, and the anxiety neuroses, and having hypoglycæmia without any demonstrable organic lesion may be due to an excess of endogenous insulin ('hyperinsulinism,' 'dysinsulinism'). Normally, after a dose of 50 gm of dextrose (Fig 28, p 125) or after a carbohydrate meal, the blood sugar at one and a half or two hours may be below the initial fasting level, it is possible that in dysinsulinism this drop in the blood sugar curve is exaggerated.

The thyroid, pituitary and adrenals all act in the opposite sense to the islets of Langerhans. An excess of secretion causes the blood-sugar curve to be raised and prolonged. Whether this is due solely to their action in mobilising glycogen from the liver and other depots, or whether also to a direct neutralising effect on endogenous insulin, is not yet known with certainty (*cf* Burn). Anyhow, hyperthyroidism (*e.g.*, exophthalmic goitre) and hyperpituitarism (*e.g.*, gigantism, acromegaly) are typically accompanied by a raised and prolonged blood sugar curve, whereas hypothyroidism (cretinism, myxædema) and hypopituitarism (dystrophia adiposo genitalis) may be associated with a normal or low normal blood

sugar curve though there are many exceptions in clinical work (Gardiner Hill *et al*) Injection of adrenaline causes hyperglycæmia and glycosuria There is no known disease ascribed to continuous hypersecretion of the adrenal medulla, but hypoadrenalism (Addison's disease) is typically accompanied by a normal or low normal blood sugar curve

(h) *The Previous Diet* In the majority of tests the previous diet is the subject's usual mixed diet, and under these circumstances when the blood sugar curve after glucose is repeated, the same or a very similar curve is secured If, however, the two curves are obtained after periods of diet which differ very greatly from one another, they may show a marked contrast In health the most extreme example is given by comparison of the curve after a prolonged fast with that after a period on full diet After the former the curve following 50 gm of dextrose will be higher than after the latter period, indeed, after the prolonged fast the dextrose ingestion may raise the blood-sugar high enough to cause glycosuria, this was well recognised many years ago, before blood sugar determinations were made, and was referred to as "hunger glycosuria" or "vagabond's glycosuria"

The point is of practical importance in disease and particularly in diabetes mellitus Blood sugar curves can only be used as a rough basis for comparing the relative severity of two cases of diabetes if in each case the diets prior to the tests have been substantially the same for several days at least Similarly, the progress of a case of diabetes, or the efficiency of any particular treatment, cannot safely be judged by simply instituting a night's fast, giving 50 gm of dextrose and following the resulting changes in blood sugar, the diet prior to the test also influences the curve, for its effect is not completely deleted by a single night's fast Clearly the amount of glycogen in store will be different on diets with very different carbohydrate contents and it would be expected that an individual with a smaller store would convert into glycogen more rapidly the 50 gm of dextrose with a resultant lower blood sugar curve The subject is complicated, and even now incompletely understood (*cf* also remarks on "afternoon glycosuria," p 128) It is probable that this influence of the previous diet has at times been responsible for the different curves reported by two investigators on the same subject, it is well recognised by biochemists (*cf* Lawrence and McCance)

Enough has been said to show that the interpretation of blood sugar curves may be anything but a simple matter It is obvious that such interpretation is impossible without full knowledge of the clinical data On the other hand such curves are often invaluable as an *extension* of the clinical examination An attempt will be made to simplify the picture

In practice there are only five groups of curves The normal, which has already been described, the renal glycosuric type, the "lag" glycosuric type, the diabetic type, and hyperglycæmic glycosuria The last four will now be discussed

## THE RENAL GLYCOSURIC TYPE OF CURVE

(Orthoglycæmic Glycosuria)

The following curve (Fig 33), obtained after 50 gm of dextrose, taken fasting, is a typical example —

Time in hours	0	$\frac{1}{2}$	1	$1\frac{1}{2}$	2
Blood sugar, mgm per 100 c c	90	150	130	80	100
Glycosuria, gm	Nil	0.3	0.5	0.1	Nil

It will be observed that the fasting level 90 is within the normal limits (80 to 120), that the peak 150 is below the usual highest point (170 to 190), and that the curve returns to the fasting level in one and a half hours. Glycæmia is normal throughout, but in spite of this there is glycosuria, this is the characteristic feature of this type of curve. The threshold may lie anywhere between 160 and 100 mgm per 100 c c, and rarely even lower. The glycosuria accordingly may be intermittent or continuous. In the above example the threshold lay somewhere between 130 and 90 mgm per 100 c c, because there was no sugar in the urine with a blood-sugar of 90 mgm but glycosuria did occur during the half hour whilst the blood sugar fell from 130 to 80 mgm.

The renal glycosuric type of curve is found in renal glycosuria and in phloridzin glycosuria. In most examples of renal glycosuria there is no evidence of any damage to the kidneys, but in a few there is

RENAL GLYCOSURIC TYPE OF BLOOD SUGAR CURVE AFTER 50 gm OF DEXTROSE

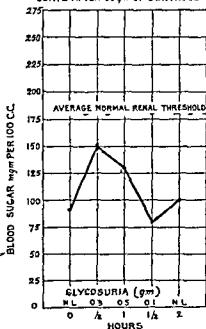


FIG 33

definite kidney disease, and it is then assumed that the renal lesion is responsible for the lowered threshold. In both types the glycosuria does no harm, and requires no treatment. The importance of establishing the diagnosis lies in the subsequent assurance of the subject that he has not got diabetes, but clearly before such an assurance is given it is essential to be absolutely certain of the diagnosis, if the subject has been on an unrestricted diet for several days (e.g., one week) beforehand, and a curve like that illustrated in Fig 33 is obtained, there can be no doubt. If, however, the curve is at or near the upper normal limit, and dietetic treatment had previously been given, more caution is required, it must be remembered that occasionally the renal threshold is lowered in diabetes mellitus, and sooner or later a case of very mild diabetes with a lowered threshold will be encountered. In cases of doubt

the subject should be given a full diet for at least a week, and then the curve should be repeated, this will generally enable a definite decision to be made. Renal glycosuria in pregnancy is discussed on p 137

## THE "LAG" GLYCOSURIC TYPE OF CURVE

### (Oxyhyperglycæmic Glycosuria—Lawrence)

MacLean described a particular variety of the raised type of curve under the heading "lag glycosuria" (Fig 34). The fasting level is normal, the blood sugar after dextrose ingestion rises very rapidly to a level obviously, but not greatly, above normal, and then falls rapidly, regaining the original level in one to two hours. MacLean suggested that there is a "lag" in the coming into play of the storage mechanism, but that once the mechanism does come into force, it is as efficient as in a healthy individual. He met with a few cases of this kind in patients who had had intermittent glycosuria for many years without apparent harm, in spite of no restriction of diet. Others have confirmed these observations, and provided that there is clear evidence of intermittent glycosuria over a period of years without any restriction of diet, the glycosuria may be regarded as harmless. Caution, however, is necessary when the glycosuria has

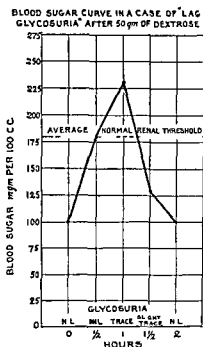


FIG 34

only recently been found, for it is possible that the case is one of "potential" or mild diabetes, in such circumstances it is wise to keep the subject under observation on a full diet, and to repeat the blood sugar curve after an interval of, say, six months, if the clinical condition and the curve remain unaltered, the glycosuria may be regarded as harmless. It is still difficult at times to assess the exact significance of lag glycosuric curves, and will be until many more cases have been kept under observation for long periods. Cases have been encountered who show the lag curve, but who exhibit glycosuria only during the test (i.e., after glucose), and not after their ordinary mixed meals. Rapid intestinal absorption may be the cause of these curves (cf Lawrence, 1936), they are often observed after gastro enterostomy and in duodenal ulcer

## THE DIABETIC TYPE OF CURVE

The following curves (Fig 35), obtained after 50 gm of dextrose taken fasting, are typical examples —

Time in hours	0	$\frac{1}{2}$	1	2	3
Blood sugar, mgm per 100 c c	160	240	260	200	170
Glycosuria, gm	Nil	1.2	2.5	1.2	Nil
Time in hours	0	$\frac{1}{2}$	1	2	3
Blood sugar, mgm per 100 c c	120	180	200	150	120
Glycosuria, gm	Nil	Nil	1.2	0.3	Nil

Examining these curves in the same way as the previous ones,

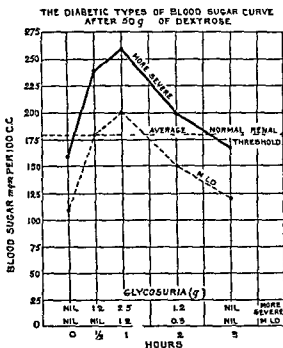


FIG 35.

it will be noted in the first (the upper one) that the fasting level is raised, that the highest point is above the normal maximum, that the curve is prolonged to the right, the normal level not being reached in three hours and that there is glycosuria. Two deductions may be made from this curve. The carbohydrate storage mechanism is deficient, and the renal threshold for glucose is raised—it probably lies between 200 and 240 in this example. Examining the second curve (the lower one), the fasting level is within normal limits, the highest point is above the normal maximum, the curve is slightly prolonged to the right, and there is glycosuria. On this evidence alone it cannot be stated for certain that the patient from whom this second curve was obtained must have diabetes. Stress cannot

be laid too strongly on the importance of combining the laboratory and the clinical observations. Thus the lower curve might equally well have been obtained from a case of sepsis, or in certain infections, or in a patient with hyperthyroidism or hyperpituitarism, or in examples of increased intracranial pressure, or even in a small proportion of patients with either liver disease or simple obesity.

① The upper curve is almost diagnostic (a few cases of hyperthyroidism show such a curve), and a curve set at a still higher level would be quite diagnostic of diabetes mellitus.

The reader may wonder why such curves are termed "diabetic" in type when only those that are grossly raised are limited to diabetes. The difficulty is to find a better label. Classification in terms of renal threshold, and such phrases as "hyperglycæmic glycosuria," "orthoglycæmic (or isoglycæmic) glycosuria," and "hyperglycæmia sine glycosuria" are of no greater value, thus in diabetes the threshold may be normal, raised or lowered, and, with suitable treatment, a patient with diabetes and a lowered threshold may exhibit "orthoglycæmic glycosuria."

### HYPERGLYCÆMIA SINE GLYCOSURIA

The following curve, obtained after a breakfast restricted in carbohydrate, is an example (Fig 36) —

Time in hours	0	1	2	3	4
Blood sugar, mgm per 100 c c	180	220	240	240	230
Glycosuria	Nil	Nil	Nil	Nil	Nil

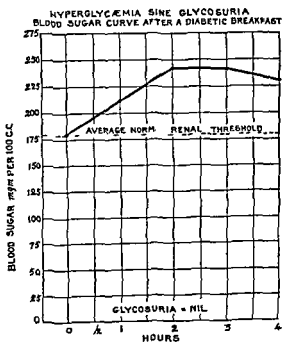


FIG 36

It will be observed that the fasting level is raised, that the highest point is above the normal maximum, that the curve is much prolonged—over four hours, and that, in spite of hyperglycæmia, there is no glycosuria. The renal threshold is, therefore, above 240 mgm. This curve was obtained from a case of diabetes under treatment by careful dieting (without insulin). If an attempt was made with insulin to maintain the blood sugar at more normal limits, it would obviously be essential to control the treatment by blood sugar tests. Urinary examinations for sugar would be valueless for the purpose.

Hyperglycæmia sine glycosuria (Fig 36) may be demonstrated in a certain number of diabetics, particularly in cases of long standing. It is also present in some sufferers from obesity. It may be observed in some uræmics and in cases of less advanced nephritis. Finally, it is not uncommon in old age to obtain curves approximating to this type, as already mentioned.

### THE BLOOD-SUGAR IN NORMAL PREGNANCY

Glycosuria, without any symptoms of diabetes mellitus and without the subsequent development of that disease, has been recognised for many years as occurring not uncommonly in normal pregnancy. Its incidence is difficult to establish, but was 5 per cent—in 640 unselected cases examined by Williams and Wills. All investigators are agreed that one factor responsible for this glycosuria is a lowering of the renal threshold, which is very common in pregnancy (60 per cent, Williams and Wills), such lowering is generally slight, and in many of the women does not cause glycosuria after ordinary meals, but does after the glucose of the tolerance test. In addition to the lowered threshold, Williams and Wills have shown that the curve in some of these women is definitely raised and may be prolonged, as compared with the usually accepted standards, though none can be regarded as diabetic, some may fairly be labelled "lag glycosuria", others must be regarded either as having a slightly lowered glucose tolerance, or as normal, in which case the range of normal must be wider than that generally accepted (compare remarks on p. 128 on incomplete knowledge of range of variation in health). This "raising" of the curve is regarded by Williams and Wills as a more potent cause of the glycosuria than the lowering of the threshold, the initial fasting level in almost all the women was normal (below 120 mgm per 100 c.c.).

At the termination of pregnancy the threshold returns to its previous level, and glycosuria ceases in the majority, when it does not, it is probably due to the persistence of the renal glycosuria or the lag glycosuria which the woman possessed before pregnancy. In short, if it be accepted that pregnancy lowers the renal threshold for glucose, this, added to the pre-existing state of "orthoglycæmia," lag glycosuria or renal glycosuria, or of diabetes mellitus, hyperthyroidism, etc., may be sufficient to explain the great variations observed in the sugar of the blood and urine in pregnancy.



## VON GIERKE'S DISEASE

(Glycogen Accumulation Disease)

This is a rare disease of childhood in which there is a great accumulation of glycogen in the liver and kidneys. The liver is greatly enlarged, smooth and not tender, there is no enlargement of the spleen and no jaundice or ascites. The fasting blood sugar is low, often in the region of 50 mgm per 100 c c, and an injection of adrenaline either fails to raise it, or does so to a much lesser degree than in a healthy child of the same age. In some of the reported cases the blood sugar curve after an appropriate dose of glucose (e g, 1 gm per kgm) is a little raised and prolonged. There is usually ketonuria, which has several times been noted to persist throughout a glucose tolerance test. The clinical and biochemical findings may be ascribed to a slight persistent carbohydrate starvation due to excessive deposition of glycogen, it has been suggested that there is a persistence of the formation of the foetal type of glycogen which is said to be more stable than that normally laid down after birth. Naish and Gumpert's paper may be consulted for references.

## METHODS OF ESTIMATING THE CONCENTRATION OF SUGAR IN THE BLOOD

Many methods have been devised for estimating the blood sugar. Of these the following are most commonly employed: Folin and Wu, Hagedorn and Jensen, Shaffer Hartmann, MacLean. Numerous modifications have been introduced in each of these. The method of Folin and Wu is useful as part of Folin's "system of analysis" and will therefore be described. The Shaffer Hartmann technique may also be applied to the tungstic acid filtrate. But for most clinical work methods applicable to capillary blood obtained by pricking the skin are preferred. For this reason the two following will also be described: Hagedorn and Jensen's 0.1 c c of blood, Folin and Wu's, as adapted by Wallis and others for 0.1 c c of blood.

For clinical purposes the selection of the method is mainly a question of personal preference, though Benedict's picric acid method has largely fallen into disuse. In most methods, not only glucose, but also non glucose reducing substances are estimated, but the former constitutes by far the larger portion (see later). For clinical purposes it is advisable to select one to the exclusion of other methods, so that the results are comparable. In all published work the method should be stated.

For routine clinical use the writer prefers the method of Folin and Wu adapted for small quantities of capillary blood simply on account of its simplicity and speed. It is important to remember however, that it gives slightly higher values in insulin hypoglycaemia than the method either of MacLean or of Hagedorn and Jensen. These last two methods give blood sugar values which approximate

closely to "true glucose" values. In MacLean's method the solutions are easy to prepare, but the special heating apparatus is troublesome, it has been given up in most laboratories, and is omitted from this edition. In Hagedorn and Jensen's method the purification of the ferricyanide is troublesome, but given the best chemicals the method is simple, and is now very widely employed either in its original form or in one of its modifications (Somogyi, Folin).

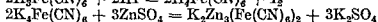
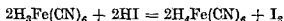
There are two stages in all the methods (1) the removal of proteins, and (2) the estimation of the reducing substances in the protein free filtrate

### Method of Hagedorn and Jensen

**Principle** The proteins are precipitated by zinc hydroxide and heating

The protein free filtrate is heated under standard conditions with alkaline potassium ferricyanide. Some of the ferricyanide is reduced to ferrocyanide by the sugar. Atmospheric re-oxidation of this ferrocyanide is prevented by its precipitation with zinc sulphate. The quantity of ferricyanide left over is determined by adding an excess of iodide and acidifying. Iodine is liberated in proportion to this ferricyanide, and is determined by titration with thiosulphate.

A blank test is performed to measure the total iodine formed by oxidation of iodide by all the ferricyanide except a trace which is reduced by impurities in the reagents. The "thiosulphate deficiency" (blank less titration value of blood filtrate) corresponds to the sugar in the filtrate used and the blood sugar is calculated with the aid of a table compiled from analyses under identical conditions of pure dextrose solutions.



**Solutions** (1) N/10 caustic soda, prepared weekly by dilution of a 2N solution

(2) Stock 45 per cent  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , from which each week a 0.45 per cent solution is prepared by diluting 1 in 100

(3) Alkaline potassium ferricyanide

{ Potassium ferricyanide	1.65 gm.
{ Fused sodium carbonate	10.6 "
{ Water	to 1,000 c c

The solution should be stored in a dark coloured bottle in the dark. It will also be more stable if the bottle is capped with a glass cover, so that dust does not get on to the stopper and neck.

(4) Iodide sulphate chloride solution

{ $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	10 gm
{ NaCl	50 "
{ Water	to 200 c c

Weigh out 1 gm of potassium iodide in each of a series of test-tubes. Add the contents of one tube (1 gm KI) to 40 c c of the above solution as required. The complete solution, including iodide, usually will not keep for more than two or three days without oxidation of the iodide to iodine. Traces of free iodine can generally be removed by filtration through thick paper.

(5) Three per cent v/v acetic acid solution

(6) Starch solution

One gramme of soluble starch is made into a paste with about 10 c c of saturated sodium chloride solution, and is poured into about 85 c c of the saturated salt solution which has been brought to the boiling point. The mixture is cooled, transferred to a

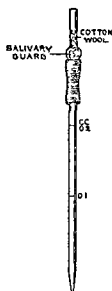


FIG 37 Blood pipette with salivary guard

measuring cylinder with a few cub centimetres of water and made up to 100 c c

(7) N/200 sodium thiosulphate prepared by dilution of an N/10 solution each day. For preparation and standardisation of the N/10 solution see Appendix. Alternatively an approximately N/200 solution is prepared and standardised against N/200 iodate on the day of the test (see Appendix).

All chemicals must be of the highest purity. The potassium ferricyanide must be specially recrystallised (see Appendix). The zinc sulphate, sodium chloride, potassium iodide and acetic acid must each be tested and shown to be iron free. The iodide must be free from iodate. As a final check mix 3 c c of iodide sulphate chloride solution, 2 c c of the acetic solution and 2 drops of starch solution when no blue colour should be obtained. Then add 0.01 c c of the alkaline potassium ferricyanide solution when the mixture should show a faint but definite blue tint.

**Technique** In an ordinary test tube (6 in  $\times$   $\frac{5}{8}$  in) place

- { 1 c c of N/10 NaOH
- { 5 c c of 0.45 per cent  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (1)
- { 0.1 c c of blood

The blood is measured in a special blood pipette which must be accurately calibrated (see Appendix) "to contain," and is best provided with a salivary guard (Fig 37). It is delivered into the zinc hydroxide suspension and washed out with that suspension the pipette being finally emptied as completely as possible by blowing through it.

Heat the tube in a boiling water bath for three minutes. Filter through a 7 cm No. 41 Whatman paper (double acid washed paper) into a boiling tube. Wash the tube and the precipitate twice with 3 c c of hot distilled water (previously measured into tubes placed in the boiling water bath till required). When the filter has drained remove the paper and touch off the last drop in the funnel stem against the side of the tube.

Add 2 c c of alkaline ferricyanide solution, mix, and place in a boiling water bath for fifteen minutes. Cool, add 3 c c of iodide sulphate chloride solution and 2 c c of 3 per cent acetic acid. Mix and stand for one or two minutes. Titrate with N/200 sodium thiosulphate from a 2 c c microburette, until the colour is pale yellow. Then add 2 drops of starch solution and complete the titration.

Perform a blank test along with the above by carrying through the above process but omitting the blood. When the solutions are fresh the blank titration should be above 1.90 c c. Provided that the N/10 NaOH and the 0.45 per cent zinc sulphate are not more than a week old a blank lower than 1.90 is probably due to the ferricyanide losing strength. Fresh ferricyanide should be made up if the blank falls below 1.80 c c of N/200 thio.

**Calculation** From the table overleaf read off the values of blank and unknown in terms of glucose per 100 c c. The unknown less blank gives the blood sugar content.

**Example** The thiosulphate was exactly N/200. Titration of blank required 1.94 c c, of unknown 1.33 c c of thio.

Blank 1.94 c c corresponds to 10 mgm of glucose per 100 c c

Unknown 1.33 c c corresponds to 119 mgm of glucose per 100 c c

Blood sugar, therefore, is  $119 - 10 = 109$  mgm of glucose per 100 c c

Alternatively a fairly accurate calculation may be made (for blood sugar values below 250 mgm per 100 c c) by multiplying the thio deficiency by 177. Thus in the example already given thio deficiency equals blank less unknown titration figure or  $1.94 - 1.33 = 0.61$ , which, multiplied by 177, gives a blood sugar of 108 mgm per 100 c c.

<sup>1</sup> The mixture of 5 c c of 0.45 per cent  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  and 1 c c of N/10 NaOH is neutral or very slightly acid to litmus paper and contains an excess of zinc sulphate.

*Relation of c c of N/200 Thio used to Glucose mgm per 100 c c*

Thio c c	0 00	0 01	0 02	0 03	0 04	0 05	0 06	0 07	0 08	0 09
0 0	385	382	379	376	373	370	367	364	361	358
0 1	355	352	350	348	345	343	341	338	336	333
0 2	331	329	327	325	323	321	318	316	314	312
0 3	310	308	306	304	302	300	298	296	294	292
0 4	290	288	286	284	282	280	278	276	274	272
0 5	270	268	266	264	262	260	259	257	255	253
0 6	251	249	247	245	243	241	240	238	236	234
0 7	232	230	228	226	224	222	221	219	217	215
0 8	213	211	209	208	206	204	202	200	199	197
0 9	195	193	191	190	188	186	184	182	181	179
1 0	177	175	173	172	170	168	166	164	163	161°
1 1	159	157	155	154	152	150	148	146	145	143
1 2	141	139	138	136	134	132	131	129	127	125
1 3	124	122	120	119	117	115	113	111	110	108
1 4	106	104	102	101	99	97	95	93	92	90
1 5	88	86	84	83	81	79	77	75	74	72
1 6	70	68	66	65	63	61	59	57	56	54
1 7	52	50	48	47	45	43	41	39	38	36
1 8	34	32	31	2°	27	25	24	22	20	19
1 9	17	15	14	12	10	8	7	5	3	2

### Method of Folin and Wu

**Principle** The proteins are precipitated by tungstic acid

The protein free filtrate is heated with an alkaline cupric tartrate solution under standard conditions. It is then treated with a solution of phosphomolybdic acid, which is reduced in proportion to the amount of cuprous salt, and, therefore, in proportion to the quantity of sugar. The compound formed by reduction of phosphomolybdic acid is blue, and the intensity of this colour is compared in a colorimeter with that of a standard solution of pure dextrose similarly treated.

- Solutions (1) Ten per cent sodium tungstate ( $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ )  
 (2)  $\frac{2}{3}$  N sulphuric acid  
 (3) Alkaline copper solution

Dissolve 40 gm of anhydrous sodium carbonate in about 400 c c of water and transfer to a 1,000 c c flask. Add 7.5 gm of tartaric acid and wait till this has dissolved. Then transfer quantitatively to the flask 4.5 gm of crystalline copper sulphate which has been dissolved in about 100 c c of water. Mix and make up to volume. A sediment often forms in time, in which case decant the clear supernatant solution.

- (4) Phosphomolybdic acid solution

✓ Dissolve 35 gm of molybdic acid and 5 gm of sodium tungstate in 200 c c of 10 per cent NaOH plus 200 c c of water in a litre beaker. Boil vigorously for twenty to forty minutes so as to remove as completely as possible the ammonia present in the molybdic acid. Cool and transfer to a 500 c c volumetric flask, washing in with sufficient water to make the volume about 350 c c. Add 125 c c of 89 per cent w/w phosphoric acid (S.G. 1.75) and make up to the 500 c c.

- (5) Stock glucose solution (1 per cent)

Prepare a saturated solution of benzoic acid by adding 2.5 gm to 1,000 c c of boiling water, and allowing to cool.

Dissolve 1 gm of pure dry glucose in 100 c c of the saturated benzoic acid. This solution appears to keep indefinitely.

- (6) Glucose standard solutions (0.01 and 0.02 per cent)

These are prepared by diluting the stock 1 per cent solution 1 in 100 and 2 in 100 respectively with the saturated benzoic acid.

Technique. Precipitate the proteins as follows in —

#### Whole Blood

1 volume of whole blood  
 7 volumes of water  
 $\frac{1}{2}$  volume of sodium tungstate  
 1 volume of sulphuric acid  
 (Dilution of blood 1 in 10)

#### Plasma or Serum

1 volume of plasma  
 8 volumes of water  
 $\frac{1}{2}$  volume of tungstate  
 $\frac{1}{2}$  volume of  $\text{H}_2\text{SO}_4$   
 (Dilution of plasma 1 in 10)

Use accurate Ostwald pipettes for the blood (or plasma). Mix and stand till precipitate clumps. Filter through an acid washed filter paper (Whatman No. 41).

In a Folin's tube (Fig. 38) place 2 c c of filtrate and 2 c c of alkaline copper solution.

In two more Folin's tubes, A and B, place —

A

2 c c of 0.01 per cent glucose  
 2 c c of alkaline copper solution

B

2 c c of 0.02 per cent glucose  
 2 c c of alkaline copper solution

These are the standards.

In each case mix and place in a boiling water bath for exactly six

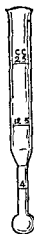


FIG. 38 Folin's sugar tube

minutes Cool for one or two minutes only, and without shaking (to avoid reoxidation of cuprous oxide by air)

To each tube add 2 c c of phosphomolybdic acid solution, and when the cuprous oxide has dissolved dilute to the 25 c c mark with water and mix thoroughly

Compare the unknown in a colorimeter, with the standard A or B, whichever it most nearly matches in colour

Calculation (Standard A)

Let S be reading of standard, U of unknown Let  $x$  be mgm of glucose in the 2 c c of blood filtrate used

The standard tube contains 2 c c of 0.01 per cent glucose

' ' 100 c c of standard A solution contain 10 mgm of glucose

$$1 \quad " \quad " \quad \frac{10}{100} \quad " \quad "$$

$$2 \quad " \quad " \quad \frac{10 \times 2}{100} = 0.2 \text{ mgm of glucose}$$

$$x \times U = 0.2 \times S$$

$$x = \frac{0.2 \times S}{U} \text{ mgm of glucose}$$

10 c c of blood filtrate are derived from 1 c c of blood

$$2 \quad " \quad " \quad " \quad " \quad 0.2 \quad "$$

$$0.2 \text{ c c of blood contains } \frac{0.2 \times S}{U} \text{ mgm of glucose}$$

$$.. \quad 1 \quad " \quad " \quad \frac{0.2 \times S}{U \times 0.2} \quad "$$

$$100 \quad " \quad " \quad \frac{S}{U} \times 100 \quad "$$

Using standard B

$$\text{Blood sugar equals } \frac{S}{U} \times 200 \text{ mgm per 100 c c}$$

Method of Folin and Wu for 0.1 c c of Blood

(Micro Folin or Wu method)

Principle As in original method

Solutions

- |   |                         |
|---|-------------------------|
| (1) Ten per cent $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ | } As in original method |
| (2) $\frac{2}{3} \text{ N } \text{H}_2\text{SO}_4$                  |                         |
| (3) Alkaline copper   |                         |
| (4) Phosphomolybdic acid  |                         |
| (5) Stock glucose solution (0.1 per cent)                           |                         |

Prepare a saturated solution of benzoic acid by adding 2.5 gm to 1000 c c of boiling water and allowing to cool

Dissolve 0.1 gm of pure dry glucose in 100 c c of the saturated benzoic acid solution This will keep almost indefinitely

(6) Glucose standard solution (0.005 per cent)

Dilute 5 c c of the stock glucose solution with saturated benzoic acid in a 100 c c volumetric flask

**Technique**

In a small test tube place  
(about 20 × 75 mm externally)  $\left\{ \begin{array}{l} 3.5 \text{ c.c. of distilled water} \\ 0.1 \text{ c.c. of blood} \\ 0.2 \text{ c.c. of 10 per cent sod tungstate} \\ 0.2 \text{ c.c. of } 2/3 \text{ N sulphuric acid} \end{array} \right.$

Mix Stand ten minutes or until protein precipitate clumps Filter through acid washed filter paper (7 cm Whatman No 41)

In a Folin's tube place  $\left\{ \begin{array}{l} 2 \text{ c.c. of blood filtrate} \\ 2 \text{ c.c. of alkaline copper solution} \end{array} \right.$

In two more Folin's tubes A and B, place —

A	B
2 c.c. of 0.005 per cent glucose	1 c.c. of distilled water
2 c.c. of alkaline copper solution	1 c.c. of 0.005 per cent glucose
	2 c.c. of alkaline copper solution

These are the standards

In each case mix and place in a boiling water bath for exactly six minutes. Cool for one or two minutes only, and without shaking (If cooling is prolonged there is a risk of oxidation of cuprous oxide by air)

To each tube add 2 c.c. of phosphomolybdic acid solution, dilute to the 12.5 mark with water, and mix thoroughly. Allow to stand for a few minutes until  $\text{CO}_2$  has escaped, otherwise bubbles may get on to the under surface of the plungers, or adhere to the bottom of the cups, and spoil the colorimetric readings.

Compare the unknown with the standard A or B, whichever it most nearly matches in colour.

**Calculation** Let the unknown be placed in the left hand cup at 40 mm. Let  $S$  be the reading of the standard which is placed in the right hand cup and moved till it matches the unknown. Then —

With standard A

$$\text{Blood sugar} = \frac{S}{40} \times 0.1 \times 2,000 = 5 \times S \text{ mgm per 100 c.c.}$$

With standard B

$$\text{Blood sugar} = \frac{S}{40} \times 0.05 \times 2,000 = 2.5 \times S \text{ mgm per 100 c.c.}$$

**Calculation from First Principles (Standard A)** Let  $S$  be reading of standard U of unknown. Let  $x$  be mgm of sugar in the 2 c.c. of blood filtrate taken.

The standard tube contains 2 c.c. of 0.005 per cent glucose



100 c c of standard solution contain 5 mgm of glucose

1 " " " contains  $\frac{5}{100}$  mgm of glucose

2 " " " contain  $\frac{5 \times 2}{100} = 0.1$  mgm of glucose

$$x \times U = 0.1 \times S$$

$$x = \frac{0.1 \times S}{U} \text{ mgm of glucose}$$

4 c c of blood filtrate are derived from 0.1 c c of blood

2 " " " " " 0.05 "

0.05 c c of blood contains  $\frac{0.1 \times S}{U}$  mgm of glucose

1 " "  $\frac{0.1 \times S}{U \times 0.05}$  "

100 contain  $\frac{0.1 \times S \times 100}{U \times 0.05}$  "

$$= \frac{S}{U} \times 200,$$

and if  $U$  be set at 40 mm ,

Then 100 c c of blood contain  $\frac{S}{40} \times 200 = 5 \times S$  mgm

Similarly with standard B,

$$\text{Blood sugar} = 2.5 \times S \text{ mgm per 100 c c}$$

**Blank** A blank should be performed with each new set of reagents employed using 0.1 c c of distilled water instead of 0.1 c c of blood otherwise proceeding as above. The blank should not show more than a just detectable very pale blue tint.

**High Blood-sugars** For values exceeding 300 mgm per 100 c c , use 1 c c of blood filtrate and c of water, and allow for the dilution in the calculation.

### ESTIMATION OF TRUE GLUCOSE OF BLOOD

As indicated already, several of the methods commonly employed for estimating blood sugar give slightly higher values than the true glucose content of the blood and these are often referred to as 'enhanced glucose' values. It is not correct to calculate the true glucose by deducting the non fermentable reducing substance (some 20 to 30 mgm —as glucose—per 100 c c) from the 'enhanced glucose' value because in different methods varying proportions of this non fermentable reducing substance are included. Methods have been devised for estimating only the glucose, but these as yet have not been widely adopted in routine clinical practice. Herbert and Bourne have shown that the true glucose values of the blood

may be calculated *approximately* by making the following deductions from the observed blood sugar values —

Method	Mgm per 100 c.c. to be deducted
Original Hagedorn and Jensen (see p 139)	4
Original Folin and Wu (see p 142)	11 (9-16)
Shaffer Hartmann	20

Since the non glucose reducing substances are mainly inside the corpuscles clearly variations in the number of blood corpuscles (anæmia polycythæmia anhydræmia) will influence the number of milligrammes which should be deducted but even then the deduction would be relatively slight. It is possible that some pathological bloods may contain a greatly increased amount of non glucose reducing substance per corpuscle but Herbert and Bourne found little variation from the normal range in their series of pathological bloods.

It is mainly in the interpretation of values which are low that methods measuring only the glucose might be expected to be superior. In actual practice in a given laboratory where all analyses are made by one particular technique it does not matter much which method is selected. It is in studying in the literature the results by a variety of techniques that the difficulty arises and to make matters worse in several publications no reference is made to the method employed.

In human blood the chief substance other than glucose responsible for the reduction is glutathione which is confined to the corpuscles. Herbert and Bourne and Folin working independently both introduced methods in which this substance was removed by diluting the blood with an isotonic solution of sodium sulphate so that the corpuscles were subsequently precipitated intact with the plasma proteins. For a fuller discussion of the subject the reader is referred to the articles by the authors quoted. Herbert and Bourne's technique follows.

#### Herbert and Bourne's Method

This is simply an application of the Folin and Wu analysis to the tungstic acid filtrate of non hæmolyzed blood.

The reagents are the same as those given on p 144 with the addition of a solution of 30 per cent crystalline sodium sulphate ( $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ ) but the glucose standard is 0.01 per cent instead of 0.005 per cent.

In a centrifuge tube place {
 

3.6 c.c. of sodium sulphate solution
0.2 c.c. of blood
0.1 c.c. of 10 per cent sod tungstate
0.1 c.c. of 2/3 N sulphuric acid

Mix rapidly<sup>1</sup> and separate the fluid at once by centrifuging and pouring the supernatant fluid through a small filter paper (Whatman

<sup>1</sup> The mixture should be bright red. The corpuscles must not be hæmolyzed.

No 41, 5.5 cm.). It is possible to obtain just over 2 c.c. of filtrate, provided the paper is of the size mentioned

The subsequent treatment of the 2 c.c. of blood-filtrate and the preparation of the standards is exactly as described on p. 145, except that the concentrations of the standards are doubled

The calculation of the results is the same as given on p. 145, because, though 2 c.c. of filtrate corresponds to 0.1 c.c. of blood (instead of 0.05 c.c.), each standard contains twice as much glucose.

With standard A (2 c.c. of 0.01 per cent glucose),

$$100 \text{ c.c. of blood contain } \frac{S}{U} \times 200 \text{ mgm.}$$

With standard B (1 c.c. of 0.01 per cent glucose),

$$100 \text{ c.c. of blood contain } \frac{S}{U} \times 100 \text{ mgm. of glucose.}$$

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## CHAPTER VIII

### CHEMICAL TESTS IN DIABETES MELLITUS AND THE CONTROL OF INSULIN TREATMENT

**Books** Joslin's *Treatment of Diabetes Mellitus*

Lawrence's *The Diabetic Life*

*Insulin Its Use in the Treatment of Diabetes*, by Macleod and Campbell

Graham's *Pathology and Treatment of Diabetes Mellitus*

MacLean's *Modern Methods in the Diagnosis and Treatment of Glycosuria and Diabetes*

White's *Diabetes in Childhood and Adolescence*

"Diabetes in Children" article in Garrod, Batten, Thursfield and Paterson's *Diseases of Children*

#### INTRODUCTION

FOR purposes of description cases of diabetes will be classified according to severity. It should be noted that the apparent severity when first seen clinically will not necessarily be the same as the real severity. The real severity of a case can only be judged from the response to treatment. A patient who at first appears to have severe diabetes may respond exceedingly well whereas another patient who, when first examined clinically, appears to have a mild form, may prove resistant to treatment. The previous treatment (if any) will often influence the apparent severity of a case which has just come under observation. In other words, the chemical findings, as usual, must always be considered together with the clinical evidence.

In discussing chemical tests in diabetes it is impossible to avoid mentioning treatment, because the tests are not only required for purposes of diagnosis, but also to control the treatment. It is, however, impossible in a book of this size to discuss the great variety in the details of treatment in vogue to-day, the reader is referred to the publications of Joslin, Graham and Lawrence. The blood sugar values reported in the tables in this chapter were all obtained by MacLean's method, and all refer to capillary blood.

#### ROUTINE EXAMINATIONS IN DIABETES

The routine examination of the urine (Chapter II) will have revealed glycosuria with or without ketonuria. A rough idea of the concentration of sugar may be gained by the use of Fehling's test as described in Chapter VI. The ferric chloride test for acetoacetic acid should be performed as well as Rothera's test. If Rothera's test is positive, but Gerhardt's test is negative, there is generally no immediate risk of serious acidosis. If Gerhardt's test

149  
R+ve  
G -ve } - no immediate risk

is positive the patient should be carefully watched for clinical signs of acidosis, and whenever possible either the  $\text{CO}_2$  combining power of the plasma or the  $\text{CO}_2$  content should be estimated (Chapter IX). A positive ferric chloride test by itself is by no means a cause for alarm, but should be regarded as a warning of possible danger ✓

Tests for protein should be made, and if proteinuria be found the centrifuged deposit should be examined. The prognosis is generally worse if protein and casts are discovered ✓

An estimation of the blood sugar should be made in every case. The general principles of the application and interpretation of blood sugar tests have already been discussed in Chapter VII, but there are a few points which need elaboration in regard to diabetes mellitus. For diagnosis the requests differ with the severity of the condition.

A single estimation at any time of the day will settle the diagnosis in severe cases, for the hyperglycæmia persists throughout the twenty four hours, subsequently the determination may be repeated as a guide to treatment as described later.

In cases which are less severe and in which there is no urgent need for treatment, it is wise to obtain the blood for a single analysis about one to one and a half hours after an unrestricted meal, this postprandial blood sugar may be definitely raised (over 200 mgm), thus settling the diagnosis, whereas the fasting level or the level at a longer interval after food may be within normal limits.

If, on the other hand, the blood sugar at this time is below 200 mgm, or if in the first instance there is doubt on clinical grounds that the urinary reducing substance is due to diabetes, then it is recommended first that the nature of the reducing substance be determined (Chapter VI), and lastly that the data for a curve should be obtained (Chapter VII).

It is important to reserve the full curve for the doubtful cases, or to employ it last as an instrument of diagnosis, because it means that the patient has to have five samples of blood taken, which require two hours of his time which may be valuable, and that the analyst has to put in several hours of work, moreover, the test is relatively expensive for the patient, or for the institution, according to circumstances.

In proved diabetes undue stress should not be laid on the height of the blood sugar in assessing the severity of the disease. Though it is commonly true that the higher it is the more severe is the diabetes this is not an invariable rule. The influence of previous treatment, if any, should always be considered. Thus a figure of 500 mgm per 100 c.c. would be more serious in a patient who had already received some treatment than in a patient whose diet at the time of the test was absolutely unrestricted. The rate of fall of the blood sugar in response to treatment is of more significance than the actual degree of hyperglycæmia. Cases of recent origin, and particularly juvenile cases, tend to have marked hyperglycæmia (e.g., 400 mgm per 100 c.c. and over), which not uncommonly is rapidly reduced by dietetic treatment alone (cf. Case No. 68, p. 151). The blood sugar of long standing cases, on the other hand, may not

be particularly high when first seen (*e g*, 200 to 300 mgm), and yet strenuous treatment may be required to bring it down to normal

*Case No 68 Female Age 56 Mild diabetes Probable duration one month Qualitative restriction of diet only No insulin No fasting and no "half ration" days*

Day of Treatment	Fasting Blood sugar mgm per 100 c c	Twenty four Hours Urine	
		Glycosuria	Ketonaemia (Rothera's Test)
0	580	+++	Vsl tr
4	202	++	Tr
8	158	Vsl tr	Sl tr
11	136	0	Tr
18	90	0	Tr
22	89	0	Tr
26	108	0	Tr
34	104	0	Tr

### "POTENTIAL" DIABETES

These patients have no definite symptoms of diabetes. Usually the glycosuria is discovered during routine examination (*e g*, in life assurance work), and is mild in degree. When on a full diet there is no ketonuria. A careful clinical examination reveals no possible cause for the glycosuria and so they are labelled "diabetes," although, strictly speaking, the absence of polyuria renders the use of the term inaccurate (*diabetes* to go through). An isolated estimation of the blood sugar may demonstrate definite hyperglycaemia, but more often the diagnosis depends on the blood sugar curve (Chapter VII).

A qualitative "diet," i.e., omission of free sugar and slight restriction of starches, is usually all that is required in the treatment of these patients (*cf* Case No 68, above). After one or two weeks of treatment the blood sugar may be estimated hourly after one of the main meals to make sure that it is strictly within normal limits. These patients should be warned not to reduce their diet sufficiently to cause loss of weight, and not to reduce their carbohydrates excessively lest ketosis result. This is important, because they are not uncommonly frightened by the suggestion of diabetes, and restrict their diet too severely, with the result that at the next visit the physician finds both loss of weight and ketonuria.

and may take too serious a view of the case, thereby causing even greater mental distress: Should this have already occurred, the only thing to do is to demonstrate to the patient the mildness of his condition by placing him, temporarily at least, on a generous but accurately measured quantitative diet, *e.g.*, in the case of an adult 200 gm. of carbohydrate, 100 gm. of protein, and 100 gm. of fat, or 2,160 Calories daily. After one or two weeks of such treatment examination of the urine and a blood-sugar curve (after one of the meals) may be performed to demonstrate that the treatment is satisfactory. It has been suggested that insulin might be used as a "prophylactic" in "potential" diabetes. Whether it would be of value is an open question. In most cases the extra demands made on both patient and physician would scarcely seem worth while.

### MILD DIABETES

There are indefinite or mild symptoms of diabetes. The urine contains as a rule small or moderate amounts of dextrose, and there may or may not be ketonuria, but no clinical symptoms of

*Case No. 64. Male Age 29. Mild diabetes of recent onset*  
*Quantitative dieting No insulin No fasting and no "half-ration" days*

Day of Treatment	Fasting Blood sugar mgm per 100 c.c.	Twenty four hours urine		Body weight without clothes		Daily Diet			Calories per diem.	Calories per kilo
		Glycosuria gm	Ketonuria Rothera's Test	lb	kgm	C	P.	F.		
0	360	++++	+++	105	47.7	36	75	112½	1,500	31
1	208	+++	++							
2	188	55	++							
4	174	42	++	106	48.2	36	75	112½	1,500	31
5	171	30	++							
7	146	17	++							
8	140	16.4	++	109	49.5	36	75	112½	1,500	30
9	139	9.4	++							
10	126	3.4	+							
11	128	V. sl tr	+							
12	119	0	Trace	110	49.9	36	75	112½	1,500	30
14	115	0	Trace							
16	88	0	V. sl tr	109	49.5	36	75	112½	1,500	30
19	108	0	Sl. tr.							
23	90	0	Trace	110	49.9	36	75	112½	1,500	30
29	88	0	Sl. tr.	110	49.9	36	75	112½	1,500	30
33	83	0	Sl. tr.							
37	109	0	V. sl tr.	110	49.9	49	75	127½	1,690	34
42	116	0	V. sl tr.	109	49.5	49	75	127½	1,690	34
44	119	0	Trace							
56	103	0	Trace							
91	104	0	Trace							
135	—	0	Trace	110	49.9	49	75	127½	1,690	34

*Notes* The ferric chloride test for acetoacetic acid was negative on and after the first day of treatment. Nowadays a higher proportion of carbohydrate and a lower proportion of fat is generally used in treatment, but this table still serves to illustrate the effect of quantitative dieting alone on the sugar in blood and urine.

**acidosis** If the blood sugar be estimated one to two hours after a full mixed meal there is definite hyperglycemia (greater than 200 mgm per 100 c c) In some cases the night's fast may suffice to bring the early morning blood sugar down to within normal limits (80 to 120 mgm per 100 c c) It is in mild cases like these that an estimation of the blood sugar before breakfast is often unsatisfactory as an aid to diagnosis In any case of doubt the diagnosis should be settled by making a blood sugar curve after 25 to 50 gm of dextrose (see Chapter VII)

Mild diabetics will respond satisfactorily to dietetic treatment without insulin That is to say, if they are placed on the minimum diet sufficient to maintain weight and permit light work, their urine will become free from sugar and their blood-sugar will fall to a normal level (*cf* Case No 64)

It is necessary to estimate the blood sugar at the beginning in order to establish the diagnosis After that it is not essential to make the estimation again until the urine has become free from sugar, unless the renal threshold for dextrose be subnormal A lowered threshold is sometimes found in diabetes, but is uncommon, in such a case, of course, glycosuria will persist after the blood sugar has become normal When the urine has become free from sugar, an estimation of the fasting blood sugar is made every third day or so until it has fallen to below 120 mgm per 100 c c Until the fasting level is normal there is no point in determining the influence of food on the blood sugar When that stage has been reached, a blood sugar curve may be made after one or more of the prescribed meals (see table below) If the blood sugar never exceeds 160 mgm per 100 c c after meals, and if it falls below 120 mgm some three or four hours after each meal it is satisfactory If the patient's clinical condition is good and his weight is stationary or rising

*Blood sugar (mgm per 100 c c) after the meals of the prescribed diets Case No 64*

Day of Treatment	23rd	29th	39th
Fasting	90	88	116
1 hour after breakfast	97	103	150
2½ hours        "	100	102	118
1½ hours after lunch	103	106	135
3       "       "       "	90	103	124
4½       "       "       "	110	117	—

*Note* Lunch was taken two and a half hours after breakfast



slightly, the diet may be regarded as suitable. This same intake of carbohydrate, protein, fat and calories should be unaltered for two or three months, when it may be increased slightly. Each alteration of diet should be followed, say, in a week's time, by blood and urine tests in order to demonstrate whether it is, or is not, satisfactory.

Insulin may be used in these mild cases if desired, but it is not essential. Occasionally it is useful at the beginning of treatment to shorten the "desugarising" period, but it makes the management of the case more complicated, and if the influence of quantitative dieting alone is not already known, its use may be unpleasant for the patient and occasionally dangerous. Another use for insulin in these mild cases is to permit larger diets than would otherwise be prescribed, in other words, to allow "luxuries." It is largely for the patient to decide whether the inconvenience of the injections and the increased difficulties of treatment are more than counterbalanced by the extra pleasures of the increased diet. It should be remembered, however, that 1 unit of insulin will "take care of" about 4 gm. of dextrose in these mild cases. When it is realised that this amount of sugar is yielded by  $\frac{1}{4}$  oz. of bread, it will be seen that even in mild cases insulin would have to be given in enormous doses in order to allow of anything like a full diet.

### MODERATELY SEVERE DIABETES

There are definite and typical symptoms of diabetes with marked glycosuria. Ketonuria is present, the ferric chloride test often being positive. There are no clinical symptoms of acidosis. At whatever hour of the day the blood is taken, hyperglycæmia will be found. A blood sugar curve after a dose of sugar is not necessary to settle the diagnosis. Indeed, the patient's condition may be made worse by the dextrose. In other words, the diagnosis is obvious, and is clinched by a single blood sugar determination.

Quantitative dieting should be started at once. The only question is whether or not insulin should be given. The procedure adopted will depend largely on the circumstances. In hospital practice, where blood analysis is instantly and repeatedly available, insulin is generally given so as to "desugarise" the patient as quickly as possible. In private practice, unless resource to blood analysis is available, it is generally wiser to try the effect of a quantitative diet alone, say, for a week or two. Unless the effect of this be known, it is very hard to estimate the dose of insulin required. Moreover, it is wise to see first whether the patient is able and willing to carry out the dieting accurately. Case No 66, p 156 shows what may happen with dieting alone. This patient needed and subsequently received insulin.

Case No 76, p 157, gives an example of the use of insulin at the beginning of treatment. In hospital practice it is often safe to give relatively large doses of insulin from the start. If too large a dose be given it is easy enough to "perform a back titration with glucose

Constant observation by the nursing staff and blood sugar tests are always to hand. In ordinary practice it would generally be wiser to try the effect of diet for a week or two and then to add insulin in small doses (e.g., 5 units twice a day) for a week and then to increase the insulin by a few units (5 to 10) at weekly intervals till the proper dose is determined. It is far easier to fix the diet and to adjust the insulin to the fixed diet than vice versa. So long as glycosuria persists (e.g., in all specimens collected at two or three hourly intervals) there is no risk of hypoglycaemia, except in those uncommon cases which have a low renal threshold for dextrose.

The existence of a low threshold can only be demonstrated by combined blood and urinary analyses (cf Renal Glycosuria, Chapter VII). If, therefore, glycosuria persists in spite of great improvement in the clinical condition or if an isolated estimation shows the blood sugar to be less than 160 mgm per 100 c.c. and yet to be coincident with glycosuria then it is wise to obtain data for a blood sugar curve after one of the prescribed meals. Case No 77 provides an illustration.

The patient's twenty-four hours urine usually contained slight traces of sugar and yet isolated estimations of blood sugar always fell well within normal limits. From the table it will be seen that slight glycosuria occurred during the period five to six hours after insulin and yet the blood sugar at the beginning of this period was 137 mgm and at the end 126 mgm per 100 c.c. The blood sugar oscillates irregularly after a mixed meal (cf Fig 30 Chapter VII) and may have risen higher than the initial figure in this case—perhaps to 140 or even 150 mgm at some time during the period of one hour. None the less it is almost certain that the threshold must have been lowered—it was probably set at a figure of the order of 130 to 150 mgm per 100 c.c.

*Case No 77 Male Age 10 Blood sugar curve after insulin and two meals showing a lowering of the renal threshold*

Hours after Insulin	Blood sugar mgm per 100 c.c.	URINE 1†		Remarks
		Glycosuria	Ketonuria Rothera's Test	
1½	63	—	—	
2½	60	0	Trace	
3½	55	0	+	
5	137	0	Trace	
6	126	Sl tr	+	

Note Breakfast (C 15 P 16½ F 35) was half an hour and dinner C 15 P 22 F 20½) was three and three quarter hours after 8 units of insulin

Disregarding now these cases with a lowered threshold, when the urine becomes free from sugar it is a good rule always to resort to blood sugar estimations. It is possible to be guided by hypoglycæmic symptoms, but this procedure is not without risk. Cases of diabetes with a raised threshold may have "hyperglycæmia sine glycosuria" (Chapter VII). If one is aiming at a normal level of blood sugar, it is obviously necessary to resort to blood analysis in these cases. In short, the difficulties in the use of insulin may be summed up as varying inversely with the ease of access to blood analysis.

*Case No 66. Male Age 45 Moderately severe diabetes Effect of quantitative diet of 36 gm of carbohydrate, 76½ gm of protein, 112½ gm of fat, or 1,510 Calories*

Day of Treat- ment.	Fasting Blood sugar mgm per 100 c c	TWENTY FOUR HOURS URINE			Body weight without clothes		Calories per kgm
		Glycosuria gm	Ketonuria		lb -	kgm	
			Rothera's Test	Gerhardt's Test			
0	226	+++	+++	Pos	109	49.5	30.5
1	196	+++	++	Neg	—	—	—
2	—	53.1	++	Neg	—	—	—
3	201	65.8	+	Neg	110	49.9	30
4	163	59.8	++	Neg	—	—	—
5	225	64.6	++	Neg	—	—	—
6	—	58.9	+++	Pos	108	49.1	31
7	219	48.4	++	Neg	—	—	—
8	—	56.1	++	Neg	—	—	—
9	—	57.2	++	Neg	107½	48.8	31
10	206	60.8	++	Neg	—	—	—

*Note* Both the fasting level of blood sugar and the daily excretion of glucose remained practically constant on the fixed diet, and the patient's weight dropped slightly. Insulin was obviously required and was started a few weeks later after trying the fasting treatment (Allen's scheme) which was no more successful than the above fixed diet. Two years later the patient was on a fixed intake of 35 gm of carbohydrate, 70 gm of protein and 120 gm of fat, or 1,547 Calories with 14 units of insulin in the morning and 10 units in the evening. His weight had risen to 137 lb., or 62.2 kgm. His diet thus yielded 25 Calories per kgm. on which he was fully able to do light work. His clinical condition was excellent.

Case No 76 Female Age 6 Moderately Severe Diabetes Diet fixed at 70 gm of carbohydrate, 50 gm of protein and 100 gm of fat, or 1,422 Calories daily Dose of insulin adjusted to fixed diet as a result of urine and blood analyses

Day of Treatment	Blood sugar mgm per 100 c c		24 HOURS URINE		Body weight st lb oz	Insulin units per dium
	Before Insulin	5 & 6 hours after Insulin	Glycosuria gm	Ketonuria Rothera's Test		
0	230	—	49.2	++	2 2 3	0
1	—	—	81.3	++	2 2 8	6
2	109	247	51.8+	+	2 2 9	12
3	—	192	41.0+	+	2 2 12	16
4	—	—	21.1	+	2 3 1	16
8	236	141	9.0	0	2 3 10	16
9	—	109	0	V sl tr	2 3 5	20
15	—	—	0	0	2 4 4	20
16	—	117	0	0	2 4 10	20
18	—	—	0	0	2 4 8	18
23	102	77	0	0	2 5 0	18
30	—	72	0	V sl tr	2 5 2	15
36	87	53	0	0	2 5 9	15
38	—	—	0	0	2 5 13	12
43	—	49	0	V sl tr	2 6 9	12
44	85	76	0	V sl tr	2 6 3	8
49	—	—	Tr	Tr	2 6 12	10
57	97	97	Tr	0	2 6 11	10
64	92	147	Tr	V sl tr	2 7 3	10
71	78	153	Tr	Sl tr	2 8 0	12

Notes It will be seen that the diet in Case 76 was relatively much larger than in Case 66. This was in order to permit growth.

After the thirtieth day of treatment the patient had slight hypoglycæmic symptoms. The dose of insulin had therefore to be decreased. As the patient put on weight and as presumably, the glycogen stores became fuller it became more and more difficult to avoid hypoglycæmic symptoms at one time and slight glycosuria at other times of the day. It was essential to make estimations of the blood-sugar to see that the treatment was satisfactory. These showed that in this case mere traces of sugar in the urine were accompanied by a satisfactory level of blood-sugar.

## SUMMARY OF TREATMENT OF ABOVE GROUPS

The great majority of cases met with in general practice fall into one or other of the grades described above. Treatment is not a matter of extreme urgency. In practice, in dealing with any one individual patient, it is simplest and safest to try in order the results of treatment (a) by qualitative restrictions of diet, (b) by quantitative dieting, and (c) by quantitative dieting plus insulin. The practitioner can do this himself, provided he is in a position to have occasional estimations of the blood sugar made to guide him in the various steps. By this method he can gauge the severity of the disease, and will discover whether the patient can be trusted to play his part. With this information it is relatively simple and safe to start insulin treatment (whenever the results of the previous dietetic treatment point to its being necessary or desirable). The converse method, viz., using insulin from the start, may be defended on the grounds that the disease may be more quickly got under control. But this makes treatment much more complicated and the necessity for repeated blood sugar estimations much greater, and the patient is not properly educated to realise the all important influence of diet. In most cases, no doubt, it would be perfectly safe to use insulin from the beginning, but in a few cases, owing to the fact that so little would be known as to the patient's ability to supply insulin endogenously, the exogenous insulin would result in severe and possibly dangerous symptoms of hypoglycaemia.

In the severe cases to be described in the next two sections, the practitioner would be well advised to insist upon institutional treatment at the start, unless he has much experience with insulin treatment, and has abundant facilities for repeated blood analyses.

## SEVERE DIABETES

There are typical symptoms of diabetes with a strongly positive ferric chloride test, and either clinical signs of acidosis, or some other complication, e.g., gangrene, sepsis, etc., which makes the case serious. Glycosuria and hyperglycaemia are marked. An isolated estimation of blood sugar at any time of the day suffices to clinch the diagnosis.

The immediate use of insulin is indicated. The only question is whether food is to be given or withheld. This will depend on the circumstances in each individual case. If food is given, strictly quantitative measurements must be made. It is simplest to fix the diet at, say, 20 to 25 Calories per kgm., and to adjust the doses of insulin as a result of blood sugar estimations. An estimation either of the  $\text{CO}_2$  combining power of the true plasma, or of the  $\text{CO}_2$  content, should be made on admission. If the alkali reserve is depleted, sodium bicarbonate may be administered for a day or two, but this drug should not be continued owing to the dangers of alkalosis (see Chapter IX).

When a really severe diabetic is first visited at his own home,

an initial dose of 10 to 20 units of insulin, and the institution of a complete fast, is perfectly safe during the few hours which must elapse before he can be transferred to an institution. The prompt administration of insulin in this way may make just the difference to a case on the verge of coma.

### VERY SEVERE OR COMATOSE CASES

A sample of venous blood should be taken at once for estimation of the alkali reserve and of the sugar content. Probably the urine will already have been examined, but, if not, a sample should be obtained at once, if necessary with the aid of a catheter. If clinically there is no doubt of the diagnosis, an injection of 15 to 20 units of insulin should be given immediately—preferably intravenously. If there is a doubt as to the cause of the coma, it is wiser to wait until the blood analysis is completed before giving insulin. It should be noted in this connection that the presence of glycosuria and of ketonuria is not proof positive that the coma is diabetic in origin. Ketonuria may occur in any comatose condition in a very few hours (e.g., six hours) simply because the patient has had no food. Glycosuria may be found as a result of increased intracranial pressure, etc. Thus cases of tuberculous meningitis, apoplexy, and “fractured base” may have both ketonuria and glycosuria, but the glycosuria is usually slight. The finding of marked glycosuria (e.g., exceeding 5 per cent) is strongly in favour of diabetes, but the demonstration that glycosuria is slight does not exclude diabetes, because sugar excretion may be poor owing to renal failure.

A complete fast should be instituted, together with the usual treatment of diabetic coma (e.g., abundant water, alkali warmth, etc.).

It is impossible to treat comatose patients satisfactorily without repeated estimations of the blood sugar, but where there is no doubt of the diagnosis the practitioner should always give the patient the greatest chance of recovery by making an immediate injection of 15 to 20 units of insulin after which admission to a suitable institution is arranged as soon as possible.

The dose and time of subsequent injections of insulin will depend on the clinical condition and the results of blood analysis.

Some authorities consider that there is no point in giving dextrose together with the insulin so long as there is a large excess of sugar in the blood, e.g., hyperglycæmia exceeding 250 mgm per 100 c.c. Glucose or carbohydrate in rapidly assimilable form (e.g., orange juice) should be available in case of hypoglycæmia. A dose of 5 gm of carbohydrate usually suffices to correct slight hypoglycæmic symptoms, and this should be repeated should symptoms recur. When the blood sugar is reaching normal levels, or when for any reason estimations are temporarily not available, the insulin may be “covered” by glucose. An allowance of 1 to 2 gm of dextrose per unit of insulin is generally ample in these severe cases. In the

example given below (Case No 81), on two occasions there was a question as to whether hypoglycæmia was developing. Blood was accordingly taken for analysis, and 5 gm. of carbohydrate (orange) were given to tide over the interval required to complete the estimation.

Others, however, believe that the combination of insulin and glucose has definite advantages, because it makes certain of the immediate provision of sufficient energy to stop the incomplete combustion of body proteins and fats, and therefore stops the further production of ketone bodies; also because it speeds up the oxidation of the ketone bodies already present in the body, and hastens the re-formation of the glycogen stores. Those who support these views disregard temporarily the hyperglycæmia and glycosuria until the emergency is passed, but, of course, thereafter promote the usual balance between the diet and the dose of insulin.

*Case No. 81. Female. Age 9. Admitted in coma*

Duration of Treatment	Blood sugar mgm per 100 c c			URINE			Insulin units	Diet			Body weight st. lb of
	Fast ing	After Food <sup>1</sup>		Glyco- suria	Ketonuria			C	P	F	
		(1)	(2)		Rothera's Test	Gerhardt's Test					
On admission *	652	—	—	+++	+++	Positive	0	Fast			3 1 0
40 minutes	—	—	—	—	—	—	20	"			—
2½ hours	522	—	—	+++	+++	Positive	20	"			—
4 "	490	—	—	+++	+++	"	—	"			—
5½ "	—	—	—	+++	+++	Wk. pos	20	"			—
6½ "	362	—	—	—	—	"	—	"			—
7½ "	199	—	—	+	+++	Positive	—	"			—
10½ "	126	—	—	0	+++	"	—	"			—
12½ "	—	—	—	0	+	Negative	—	"			—
1 day *	266	—	—	+++	++	Negative	6	Fast			—
2 days	—	92	237	+	+++	Positive	20+14	40	50	70	—
4 "	—	89	90	0	+	Negative	20+14	40	50	70	3 8 8
7 "	—	98	152	0	st tr	"	20+14	40	50	70	3 8 8
10 "	—	85	—	0	—	"	20+14	50	60	80	3 7 0
16 "	—	88	—	0	v sl tr	"	20+14	50	60	80	3 7 0
18 "	—	75	87	0	0	"	16+10	50	60	100	3 7 8
20 "	—	—	—	0	v sl tr	"	18+10	75	60	120	3 7 8
22 "	—	92	—	0	Trace	"	14+ 8	75	60	120	3 7 12
26 "	—	—	—	0	v sl tr	"	14+ 8	80	65	140	3 8 8
31 "	—	133	—	0	0	"	12+ 7	80	65	140	3 9 8
37 "	—	92	105	0	0	"	10+ 6	80	65	140	3 10 8
44 "	—	79	—	0	0	"	8+ 5	80	65	140	3 12 12
52 "	—	92	—	0	v sl tr	"	8+ 5	80	65	140	3 13 8
57 "	—	213	220	+	Trace	"	10+ 6	80	65	140	4 0 8
60 "	—	251	—	+	+	"	12+ 8	80	65	140	4 0 12
66 "	—	134	144	0	0	"	12+ 8	80	65	140	4 2 4
73 "	—	72	60	0	Trace	"	12+ 8	80	65	140	4 4 4
92 "	—	74	105	0	—	"	12+ 8	80	65	140	4 5 12

\* Estimations (1) approximately 6 hours after first dose of insulin and 2 hours after second meal; (2) approximately 9 hours after first dose of insulin and 2½ hours after third meal. Second dose of insulin 10 hours after first.

<sup>1</sup> Plasma bicarbonate, 18 vols per cent. Blood cholesterol 218 mgm, and blood urea 95 mgm. per 100 c c.

<sup>2</sup> Five gm. of carbohydrate (orange) whilst waiting result of analysis.

<sup>3</sup> Plasma bicarbonate, 70 vols per cent. Blood urea 54 mgm per 100 c c.

<sup>4</sup> Big toe septic.

## PREPARATION FOR SURGICAL OPERATIONS

Cases needing operation require the same tests and treatment, according to the severity of the diabetes, as outlined above. Where the operation is not urgent, the diabetic should be treated medically first, and not operated upon until the diabetes is under satisfactory control. When the operation is urgent, insulin should be used at once. Chemical tests may be important in the post-operative treatment. Acidosis may develop and its degree should be measured. In cases where a focus of sepsis has been drained, the insulin requirement may fall suddenly. Repeated estimations of blood-sugar would then be necessary to control properly the doses of insulin.

## DIABETES IN PREGNANCY

Reducing substances are not uncommonly found in the urine of pregnant women. In some cases the reducing substance is lactose (see Chapter VI). In most cases the reducing substance is glucose, but the glycosuria is of the renal type (see Chapter VII). Occasionally true diabetes and pregnancy are associated. The chemical tests and the treatment then vary according to the severity of the diabetes, but the principles are similar to those already given. A pregnant diabetic requires a relatively large diet and a greater allowance of protein. The dose of insulin generally has to be increased in pregnancy, but sometimes may have to be decreased in the later months, possibly owing to a supply of endogenous insulin from the foetus. The actual labour may be regarded as on a par with a surgical operation (see above). After labour the diabetic mother may require careful treatment and repeated estimations of blood-sugar, the supply of insulin from the foetus having been removed.

## SEPTIC COMPLICATIONS AND INTERCURRENT INFECTIONS

It is well recognised that sepsis and infections make it necessary to increase the dose of insulin. Even a cold in the head may cause a slight rise in the blood-sugar, which must be met by extra insulin.

Case No 81, p 160, the fifty seventh day of treatment, illustrates the influence of mild sepsis clearly. The patient was on a fixed intake of carbohydrate, protein and fat, and had been under perfect control with insulin for several weeks previously. One day glycosuria was noted during the routine daily laboratory examination. The possibility of the patient having broken diet was excluded. Systematic examination revealed a suppurating whitlow of the big toe. An extra 7 units of insulin were required to bring the blood sugar back to normal limits. When, with local treatment, the focus of sepsis cleared up, the extra insulin had to be discontinued to avoid hypoglycæmic reactions.

The onset of severe sepsis not uncommonly precipitates coma. Therefore, all comatose patients should be carefully searched for a focus of sepsis. Sometimes these severe cases require an enormous increase in insulin, daily doses of 200 to 1,200 units having been recorded as necessary in a few instances. It is not known why sepsis has this striking effect. As a result of work on rabbits injected



with diphtheria toxin, Lawrence and Buckley suggest that sepsis and infections may raise the insulin requirements owing to the action of the toxins on the thyroid and adrenal glands, thereby causing an increase of their internal secretions and hyperglycæmia. Whatever the true explanation may be, the obvious remedy is to increase the dose of insulin. In mild infections and mild sepsis an extra 5 to 10 units daily generally suffices. The actual dose may often be decided simply by urinary examinations for sugar. In severe cases, when large doses are necessary, resort to blood analysis is usually essential, and this is particularly important when sudden changes may occur, e.g., after drainage of an abscess.

### TUBERCULOSIS

It is an old clinical observation that when tuberculosis supervenes, the diabetes *apparently improves*. There can be little doubt that in many such cases the "improvement" was due *entirely* to *reduction* of food intake. Even nowadays there is probably not a sufficiently wide appreciation of the preponderating influence of diet in diabetes. Only those who have made accurate *quantitative* studies can realise the variations which may creep in from the use solely of lists of foods "allowed" or "forbidden."

At the same time it is wise to bear in mind that many such clinical statements, although subsequently proved not to contain a whole, often contain a partial truth. In this connection it is interesting to note that Lundberg believes he has discovered in the tissues of tuberculous patients a substance reducing the blood sugar. This needs confirmation.

The chemical tests and the plan of treatment necessary in diabetes complicated by tuberculosis are no different from those required in diabetes not so complicated, except that treatment is necessary for the tuberculosis as well as the diabetes, and that a larger diet and more insulin are required to strengthen and restore the loss in weight of a tuberculous diabetic.

### NOTES ON DIABETES IN CHILDHOOD

Most of the following points have been mentioned incidentally in previous paragraphs but may conveniently be summarised.

The chemical tests required are the same as for adults, but as a rule blood samples are taken as seldom as possible, because children are often frightened by pricks. For several reasons, however, blood analysis is often particularly important in children. Thus the blood sugar may fall very rapidly solely as the result of diet. Symptoms of hypoglycæmia are less definite in children than in adults, and such symptoms often do not occur till the blood sugar has fallen some 20 to 30 mgm below the adult "hypoglycæmic" level (Harrison). *A lowered renal threshold appears to be commoner in children than in adults*.

Children develop ketonuria more rapidly and more readily than adults (p. 178). There is consequently a greater risk of acidosis.

They require relatively larger diets to permit growth, and need a higher proportion of protein, relatively more carbohydrate to reduce ketosis, and relatively less fat to avoid ketosis and fat indigestion

Insulin is necessary in the majority, and is commonly limited to two injections daily owing to a child's natural dislike of the procedure. The doses of insulin frequently have to be relatively large

## SUGAR TESTS IN BLOOD AND URINE TO CONTROL INSULIN TREATMENT

The necessity for blood sugar determinations has already been exemplified on more than one occasion in previous sections. In brief, they are essential in establishing the preliminary balance between a fixed diet and the dose of insulin, and it is dangerous to dispense with them in treating a very severe or comatose patient

Sugar tests in both blood and urine are necessary to determine whether the renal threshold for dextrose in a diabetic is normal, lowered or raised. If the threshold is normal, subsequent examination of the urine for sugar suffices to control insulin treatment over long periods, but even in these cases it is advisable and gives confidence to check the urinary findings by an occasional estimation of the blood sugar, say, at intervals of three months.

If the threshold is low, and this fortunately is uncommon, resort to blood analysis should be more frequent, because the urine will often contain sugar and urinary examinations alone fail to give certain information of hyperglycæmia. If the threshold is raised, the urine should never contain sugar. glycosuria demands an alteration in treatment. But though aglycosuric, the patient may have hyperglycæmia, and this is considered undesirable. Blood analysis should therefore be employed more frequently in these cases also.

The main purpose of this section is to give the reader some idea of the optimum level of blood sugar in insulin treated patients. The question arises as to what is the optimum level of blood sugar in order to insure the maximum benefit from insulin medication. There are two schools of thought in this matter. "Those who aim at keeping the blood sugar normal and those who pay most attention to the clinical condition, and do not mind if the blood sugar is raised." F G Banting, W R Campbell and A A Fletcher, and F M Allen and J W Sherrill agree that the fasting value of the blood sugar should be kept as near normal as possible. E P Joslin, R M Wilder, J R Williams, R Fitz, H Geyelin, R T Woodyatt, and their co workers all seem to agree that the clinical condition comes first and that the blood sugar is a secondary matter" (*Lancet*, 1923, ii, 671). The English workers are similarly divided into two schools.

The writer has attempted to keep the blood sugar level as nearly "normal" as possible, but feels that the distinction between the two schools referred to above, is sometimes more apparent than real. The differences of opinion are based partly on matters of

definition, and partly as a result of observations on diabetics of varying degrees of severity. In the first place, what is meant by a "normal" blood sugar? Is the blood sugar of the diabetic on

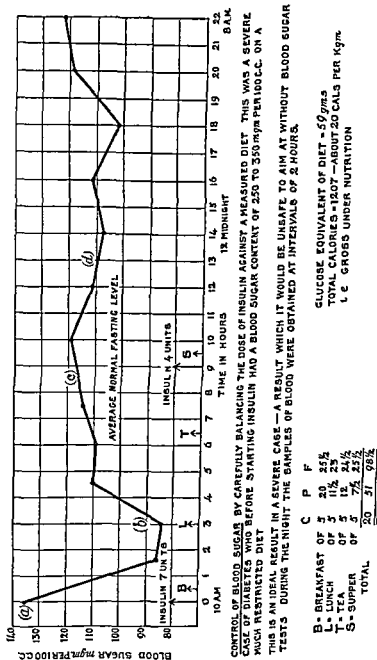


FIG 39

a restricted diet to be kept at the same level as that of a healthy individual on a similarly restricted diet, or as the blood sugar of a diabetic on a restricted diet to be allowed to fluctuate between the same figures as does that of the normal being on a full diet (or as

that of the normal being after a dose of 50 to 100 gm. of dextrose) ? (See Chapter VII.)

If a healthy individual is placed on a diet greatly restricted in

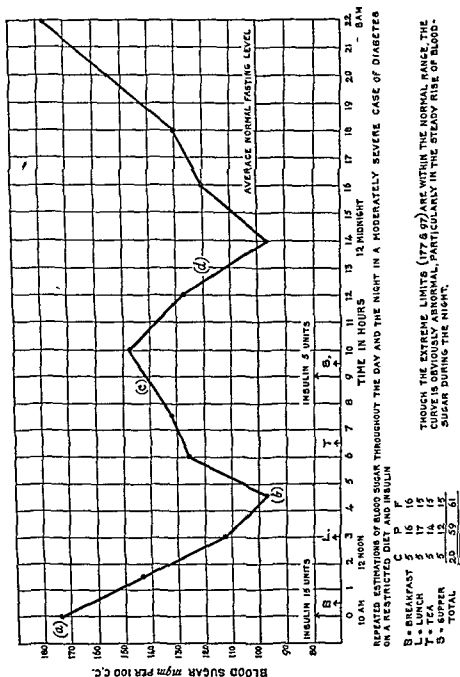


FIG. 40.

THOUGH THE EXTREME LIMITS (177 & 97) ARE WITHIN THE NORMAL RANGE, THE CURVE IS OBVIOUSLY ABNORMAL, PARTICULARLY IN THE STEADY RISE OF BLOOD-SUGAR DURING THE NIGHT.

carbohydrate his blood-sugar will rise extremely little. Thus Poulton and Payne found that their own blood-sugars varied between 80 and 120 mgm. only, on diets limited to green vegetables, protein and fat (see Fig. 2 in Poulton's paper). In other words, their blood-sugar throughout the twenty-four hours lay within the

fasting limits of healthy individuals. If this is to be taken as the desired range for diabetics *under insulin treatment*, very few would have a "normal" blood sugar throughout the twenty four hours. In *mild* cases of diabetes *without insulin* it is easy enough to keep the blood sugar within such limits (see table, p 153). When, however, insulin is given to these mild cases the diet has to be increased, and then it is not so easy to keep the blood sugar within the same limits. It can, of course, be done even in fairly severe cases, by very careful spacing of the meals with several injections of insulin and repeated estimations of the blood sugar percentage, but this is not practicable in general. As the diabetes becomes more severe it becomes increasingly difficult to obtain such a result under any conditions. Fig 39 shows that it can be done in a fairly severe case, but even here an important point emerged. Limiting ourselves as we did to two injections in the twenty four hours, we found that the only way to obtain this type of "normal" blood sugar was to reduce the diet to 20 Cals per kgm, on which the patient lost weight and strength. Obviously it was not fair to continue such a diet for more than a short time (two months).

Is it possible, then (using a restricted diet and insulin), to keep a diabetic's blood sugar within the extreme limits of a normal individual on a full diet? In the milder cases it certainly is, but in more severe cases repeated estimations for several hours show that the blood sugar curve does not strictly conform to that of a normal individual (Fig 40). In particular, with increasing severity of the diabetes, it becomes more and more difficult to obtain a normal blood sugar at the end of the night's rest unless an injection is made late in the evening or during the night, a procedure not commonly adopted (cf v Noorden and Isaac).

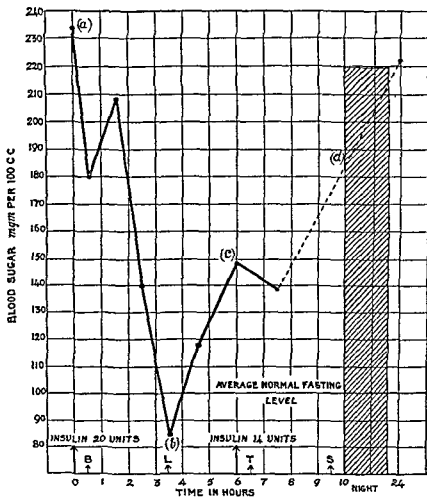
The position, then, depends on what is meant by a 'normal' blood sugar, and on the severity of the diabetes. The writer's observations lead to the following conclusions.

(1) The blood sugar in mild cases of diabetes may be made to fluctuate between levels of 70 and 130 or 140 mgm without insulin, by diet restriction alone.

(2) The blood sugar of mild to moderately severe cases may be made to fluctuate between levels of 80 and 180 by dieting and insulin, which is usually given in two doses daily. The blood sugar curves after the prescribed meals of patients treated in this way are often not normal, though the highest and lowest points are within normal limits. Thus the level after a night's fast, and between three and four hours after a meal, is often raised (see Fig 40).

(3) The blood sugar of severe cases may be made to fluctuate between 80 and 220 to 260 mgm by dieting and insulin. If two doses of insulin are used, and this is a very common practice, it will often be found that there is definite hyperglycæmia (160 to 260 mgm) first thing in the morning, and that, with increasing severity of the disease the fewer are the number of hours out of the twenty four during which the blood sugar lies between 80 and 120.

(4) More carbohydrate and less fat is used in diabetic diets nowadays, there are several dietetic methods of arriving at the same result. But when the daily quantities of carbohydrate or of total Calories are large it becomes difficult to control the blood-sugar



BLOOD SUGAR CURVE SHOWING THE TREMENDOUS OSCILLATIONS WHICH FOLLOW DIETS CONTAINING A HIGH PROPORTION OF CARBOHYDRATE

	C	P	F	
B • BREAKFAST	• 40	23	22	GLUCOSE EQUIVALENT • 201gm PER DIEM
L • LUNCH	• 27	13	17	TOTAL CALORIES • 1588 • •
T • TEA	• 60	17	25	CALS PER Kgm • 30 •
S • SUPPER	• 25	19	10	
TOTAL	152	72	72	

FIG 41.

with insulin. Under such conditions the sugar content tends to rise rapidly and to fall rapidly (Fig 41). Periods of gross glycosuria and symptoms of hypoglycæmia, in all except the milder types of the disease, tend to follow rapidly one upon the other.

In conclusion, then, of diabetics requiring insulin, an attempt is made to keep the blood-sugar between the limits of 80 and 180 mgm,

and this probably is what most workers mean when they say they aim at keeping the blood sugar "normal" In the severe cases it is often difficult to avoid an hyperglycemia of as much as 260 mgm per 100 c c for brief periods in the twenty four hours if only two doses of insulin are used, and diets adequate to maintain weight and strength are allowed

### The Best Time for Collecting Blood

In practice it will obviously rarely be possible to make such prolonged observations as are recorded in Figs 39 to 41 It is necessary to have such curves in mind, however, in order to interpret isolated observations and in order to select the best times at which to make blood analyses (*a b, c* and *d* in Figs 39 to 41)

It will be seen that the most useful data are (*a*) the fasting level before the first dose of insulin, and (*b*) the lowest level after the injection (the commonest time for hypoglycæmic symptoms) The time of the latter varies with the amount of carbohydrate in the diet and with the duration of treatment On high carbohydrate diets it usually occurs three to four hours (Fig 41), and with low carbohydrate diets three to six hours after the injection (Figs 39 and 40) After prolonged treatment the lowest point tends to be nearer six than three hours, possibly owing to the greater amount of stored carbohydrate upon which call can be made In the writer's experience, this point (*b*) is the best time of the day for estimating the blood sugar if only one test is to be made, treatment may be regarded as satisfactory if the result falls between 80 and 120 mgm per 100 c c

Otherwise isolated tests should be made to check the significance of supposed hypoglycæmic symptoms Thus, in addition to the morning point (*b*), it is not uncommon to encounter slight symptoms either shortly after the second injection of insulin, or soon after the last meal of the day An estimation just before the second dose of insulin (*c*) will often give warning of the likelihood of such symptoms An estimation late in the evening (*d*) is occasionally of value to determine whether the morning hyperglycemia has persisted throughout the night, or whether it occurs only in the early hours of the morning This last test (*d*), in other words, gives some idea as to whether the second dose of insulin is large enough

These blood sugar estimations can to some extent be replaced, and should always be supplemented by urinary tests for sugar, always assuming that the renal threshold for dextrose is known approximately (see above) If the urine be examined at intervals of two or three hours it will be easily understood that a rough idea of the shape of the blood sugar curve can be pictured

In conclusion once the diet and insulin have been satisfactorily balanced, the work entailed is not nearly so great as might be imagined from the above description The patient can often be taught to test his own urine, and, given an intelligent patient, visits

to the doctor may frequently be lengthened out from intervals of one week to intervals of three months. If the patient is not intelligent, or is unwilling to co-operate, it is dangerous to attempt to keep the blood sugar normal. Insulin is then best avoided, or employed in "safe" doses, i. e., in amounts insufficient to keep the blood sugar normal.

### THE HYPOGLYCÆMIC LEVEL

When healthy adults are given a sufficient dose of insulin, hypoglycæmic symptoms start when the blood sugar has fallen below 70 mgm, or more often below 60 mgm per 100 c c of capillary blood. It is impossible to state the hypoglycæmic level more exactly, for there are slight individual variations, and, as mentioned previously (p 146), the level varies a little with the technique employed for the estimation. At this hypoglycæmic point the venous blood sugar may be still lower, e. g., 30 mgm per 100 c c.

For subjects not receiving insulin there are quite marked differences of opinion as to what shall be termed hypoglycæmia, some investigators have labelled "hypoglycæmic" a level of blood-sugar which others would regard as within normal limits. The writer's practice (using Hagedorn and Jensen's or the micro Folin and Wu's method) is not to report hypoglycæmia until the capillary blood sugar is below 60 mgm per 100 c c. Here again further investigation of "the normal" is required. The matter is of practical importance because requests for blood sugar estimations have become more frequent in the hope of obtaining laboratory evidence that attacks of giddiness, or faintness, or an empty feeling are due to hypoglycæmia (cf "Dysinsulinism," p 131). There is a real danger to the patient of a facile but false diagnosis. If the attacks are undoubtedly stopped in a few minutes by eating carbohydrate, that is good evidence that the symptoms were "hypoglycæmic," but otherwise, unless the blood sugar is below 60 mgm, every care should be taken to exclude other possible causes of the symptoms. The best time for taking the blood is during an attack, sometimes symptoms may be provoked in about one and a half to two hours by giving 50 gm of glucose and encouraging the subject to take exercise, but in the writer's experience the usual glucose tolerance test at rest is of no help in diagnosis.

In the insulin treatment of diabetes also, symptoms commonly begin when the capillary blood sugar has fallen below 60 mgm per 100 c c, but the hypoglycæmic level varies much more in diabetes than in health. Thus symptoms have been observed when the blood sugar was 90 or 100, or even up to 150 mgm. Possibly the rate of fall is of more importance than the actual level. Lawrence states that he has often noted symptoms at a higher level of blood sugar at the beginning of insulin treatment. Reference has already been made (p 163) to the fact that the hypoglycæmic level is often lower in children than in adults.



## SUSPENSION INSULINS, PROTAMINE INSULIN ZINC PROTAMINE INSULIN

Several attempts have been made to retard the absorption of insulin and so prolong its action. The most successful of these up to 1936 was protamine insulin, introduced by Hagedorn and his colleagues, the ordinary soluble insulin hydrochloride was precipitated by the protamine (salmuridin) from the sperm of the rainbow trout, and the precipitate was made into a suspension at pH 7.3 for injection. Scott has shown that a more stable compound with a still more prolonged action is produced by the addition of zinc, and zinc protamine insulin is issued in suspension in one bottle. The quantity of zinc is so minute, 1 mgm. in 500 units of insulin, that it is not toxic.

After soluble insulin hydrochloride the blood sugar starts to fall in fifteen to thirty minutes (in the absence of food), and the insulin effect wears off in eight to ten hours. After protamine insulin the blood sugar falls more slowly, but the insulin effect lasts longer, some twelve to fifteen hours, it is therefore a common practice to give soluble insulin in the morning to prevent hyperglycemia after meals, and protamine insulin in the evening to prevent hyperglycemia during the night, and especially in the early hours of the morning. Zinc protamine insulin scarcely affects the blood sugar for three to six hours, but its action continues for twenty four hours, and still longer when the dose is big, its action is therefore to some extent cumulative, and three to six days must elapse before the full effect of a fixed daily dose can be judged. Mild and moderately severe cases of diabetes can be controlled by a single morning injection of zinc protamine insulin, but severe cases require also a morning injection of soluble insulin, which can be given in the same syringe (Lawrence and Archer). Each patient must be treated as an individual problem, and it must be remembered that suspension insulin, presumably owing to variations in rate of absorption, is less regular in action from day to day than is soluble insulin.

Lawrence and Archer suggest 30 units of zinc protamine insulin as a starting dose and adjust it up or down according to urinary and blood tests for sugar. They find it essential to give more carbohydrate late in the day than with ordinary insulin and prescribe it for afternoon tea, dinner and at bedtime. Hypoglycemia is more subtle in onset than with ordinary insulin owing to the less rapid fall of blood sugar, but the symptoms are apt to be sudden and severe when they do develop, indeed the risk of severe hypoglycemia at night is the chief difficulty of treatment with zinc protamine insulin. A striking feature of the action of the suspension insulin in severe cases under good control is the complete absence of ketonuria throughout the twenty four hours.

The above are points to be expected in the use of an insulin which is more slowly absorbed. For fuller details the reader is

referred to the articles of Lawrence and Archer, and to the papers to which they give references

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## CHAPTER IX

### KETOSIS, ACIDOSIS AND ALKALOSIS

**Books and Reviews.** Peters and Van Slyke's *Quantitative Clinical Chemistry*, Vol I, chapter on Carbonic Acid and Acid Base Balance

De Wesselow's *Chemistry of the Blood in Clinical Medicine*, Chapter II  
An excellent chapter upon which the writer has largely based his own account

*The Acid Base Equilibrium of the Blood* Medical Research Council Special Report Series, No 72

Clark's *The Determination of Hydrogen Ions*

Austin and Cullen's *Hydrogen Ion Concentration of the Blood in Health and Disease* (Medicine Monograph)

Haldane and Priestley's *Respiration*

Van Slyke's *Factors Affecting the Distribution of Electrolytes, Water, and Gases in the Animal Body* (Monograph on Experimental Biology)

Barcroft's *The Respiratory Function of the Blood*

Meakins and Davies' *Respiratory Function in Disease*

Henderson, L J *J Biol Chem*, 1921, 46, 411, and *Blood A Study in General Physiology*

Van Slyke, D D, *Physiol Rev*, 1921, 1, 141, and *J Biol Chem*, 1921, 48, 153

Wilson, D W, *Physiol Rev*, 1923, 3, 295

IN Chapter II it was recommended that all urines containing reducing substances should automatically be tested for acetone bodies. It is natural therefore to study ketosis immediately after the problems arising from the discovery of "sugar" in the urine. It should be added at once that ketonuria is often present in the absence of glycosuria. Ketosis leads to a consideration of the wider subjects of acidosis and alkalosis.

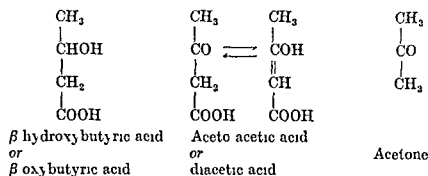
### KETOSIS

The term "acidosis" was originally applied to those conditions in which acetoacetic and  $\beta$  hydroxybutyric acids or their salts were excreted in the urine. Subsequently it has been shown repeatedly that the excretion of these bodies is *not necessarily* accompanied by a truly acid reaction of the urine. In fact, in certain cases the reaction of the urine may be alkaline at a time when ketonuria exists. It was, therefore, obvious that the original definition of "acidosis" was misleading. Moreover, the name "acidosis" was later extended to include an increase of acids other than acetoacetic and  $\beta$  hydroxybutyric, or a lowered bicarbonate content of the blood. Considerable confusion has resulted from these different definitions of "acidosis". The modern attitude with regard to the presence of acetone bodies in blood or

urine may be summarised as follows. When abnormal quantities of aceto acetic acid, or of  $\beta$  hydroxybutyric acid, or of their salts, or of acetone (a decomposition product of aceto acetic acid), are present in the body fluids or urine, the term "ketosis" is applied. "Ketonæmia" signifies an excess of these bodies in the blood, "ketonuria" an excess of the same in the urine. By this means any suggestion as to reaction is avoided. Ketonæmia may be accompanied by either a normal blood reaction, or by a shift either to the acid or to the alkaline side of normal. Similarly ketonuria may be coincident with a neutral, acid, or alkaline urine. In other words, if tests for acetone bodies are positive, it is convenient to use the single word "ketosis," instead of saying that these abnormal bodies have been found. It is, after all, only a matter of words, or, rather, of avoiding terms that suggest conditions which may not exist in fact.

This question of definition of terms will be considered further in the next section.

The acetone bodies are three in number —



Acetone may be excreted both in the breath and in the urine. It is probably derived from aceto acetic acid by loss of  $\text{CO}_2$ . Aceto acetic acid and  $\beta$  hydroxybutyric acid or their salts are excreted in the urine.

It will be noted that two formulæ are given for aceto acetic acid. The second of these represents the acid as an unsaturated body containing a double bond, and the "enolic" grouping



The correct name for the compound having this second formula is  $\beta$  hydroxycrotonic acid. It is believed that these two isomers, true aceto acetic acid and  $\beta$  hydroxycrotonic acid, are readily interconvertible, and that in the body they or their salts exist in a state of dynamic equilibrium. The point is of practical importance because it has been suggested (Hurtley) that the danger of ketosis lies in the poisonous action of this second unsaturated compound.

or its salts. On this hypothesis ketosis should be treated on the basis of the *poisonous* action of this isomer.

The *acidic* properties of aceto acetic and  $\beta$  hydroxybutyric acids need cause no alarm until the alkali reserve of the body has been seriously depleted. In short, ketosis should be *prevented* as far as possible by supplying a sufficiency of carbohydrates, and, if necessary, insulin. Alkalies, *e.g.*, sodium bicarbonate, are useful in the later stages in preventing a depletion of the alkali reserve, but they have little or no influence on the poisonous action of the isomer of aceto acetic acid. In fact, it is important further, to remember that excessive administration of alkali may actually be dangerous, in that the dangers of alkalæmia may be superimposed on the dangers of ketonæmia.  $\beta$  hydroxybutyric acid does not contain an enolic grouping and is not poisonous. In fact, it has been suggested (Hurtley, 1916) that one of the actions of the liver in ketosis is the conversion of the poisonous isomer of aceto acetic acid into the less harmful  $\beta$  hydroxybutyric acid.

### Qualitative Tests for Acetone and Aceto-acetates in the Urine

In clinical work Rothera's and Gerhardt's are the two tests most commonly employed.

**Rothera's Test for Acetone and Aceto-acetic Acid.** Both acetone and aceto acetic acid give with sodium nitroprusside in ammoniacal solution a purple or "permanganate" colouration. Saturation with ammonium sulphate removes a number of interfering substances thereby making the test more delicate. A brief outline of a convenient technique has been given in Chapter II.

The reaction is very delicate for both ketones. It will detect 1 part of acetone in 20,000 and 1 part of aceto acetic acid in 400,000 parts of water (Hurtley, 1913). Applied to urine, it is less sensitive than for simple aqueous solutions, but the limit of sensitivity may vary with the technique and particularly with the proportion of sodium nitroprusside used when looking for minimal amounts of acetone bodies. Using undiluted urine (or ketone containing urine diluted with normal urine), the method can detect 1 part of acetone in 10,000 parts, and 1 part of aceto acetic acid in 125,000 parts. Using urine diluted with water, naturally the limit of sensitivity approaches that for aqueous solutions. The test is clearly much more sensitive for aceto acetic acid. Since however, acetone is derived from aceto acetic acid, the two substances have the same significance clinically, and it is valuable to have a test which responds to both. On the other hand, Rothera's test is almost too sensitive for clinical work and positive reactions have not infrequently caused unnecessary alarm. The rate of development of the colour gives a rough indication of the degree of ketonuria, and with practice it is easy to gauge when really excessive quantities of acetone and aceto acetic acid are present. Another simple expedient consists in diluting the urine till a positive reaction is no longer obtained. The degree of dilution required gives a rough measure of the quantity of acetone bodies.

Rothera's reaction is signally free from difficulties. The writer and also Dr R. D. Lawrence (personal communication), has however, occasionally noticed a very similar colour reaction of unknown cause. When the urine has been treated with ammonium sulphate and sodium nitroprusside the addition of even a few drops of concentrated ammonia solution produces a purplish red or bluish purple colour, which develops more quickly and fades more quickly than the corresponding intensity of colour due to ketonuria. The reaction can be differentiated by first thoroughly boiling the urine which does not destroy it; moreover Legal's test for acetone and aceto acetic acid is negative (about 5 c.c. of urine treated with several drops of a concentrated solution of sodium nitroprusside and then made alkaline with NaOH turns ruby red to become a deeper red on acidification with acetic acid if the ketones are present but yellow if the unknown substance only is present just as in the case of normal urine). The colour reaction becomes less after keeping the urine for two days and is not due to a drug, it has been observed in the urines of both diabetic and non-diabetic individuals.

**Gerhardt's Test for Aceto-acetic Acid** (see also Chapter II)  
 Ferric chloride yields a Bordeaux red colour with aceto acetic acid. On adding a 10 per cent solution of the ferric chloride to a 1 in column of urine in a test tube drop by drop, a white precipitate of ferric phosphate is first seen. Addition of the iron solution should be continued till the maximum precipitate of phosphates has been obtained. The mixture is then filtered and a further drop or two of ferric chloride is added to the filtrate till the maximum red colour is obtained, but excess of ferric chloride should be avoided lest the red colour be masked. It is safest to disregard orange and orange brown tints, since substances other than aceto acetic acid may be responsible for these.

The drugs which interfere with this test are salicylates, and the bodies excreted after the administration of aspirin (acetyl-salicylic acid), thallin, antipyrine (phenazone), etc. The tint obtained with salicylic acid is more a purple than a Bordeaux red, but the colour obtained after the administration of phenazone is very similar to that obtained with aceto acetic acid. A positive test due to these drugs is easily differentiated from one due to aceto acetic acid, by observing the effect of boiling. On boiling, aceto acetic acid is decomposed into acetone and  $\text{CO}_2$ , whereas the excretion products of the drugs are unaffected. It is important to note, however, that simply heating a quantity of urine to the boiling point in a test tube of the usual bore (about  $\frac{5}{8}$  in.) does not suffice to complete this decomposition. It is necessary to boil the urine vigorously for several minutes in a large boiling tube or in an open vessel. After such treatment the reaction will remain positive if due to the drugs, but it will become negative if aceto acetic acid had originally been responsible for the red colour. A positive ferric chloride reaction must be due to drugs if the urine gives a negative response to Rothera's test.

Gerhardt's ferric chloride reaction is given by aceto acetic acid, but not by acetone. It is not nearly so sensitive a test for aceto-acetic acid as is Rothera's reagent. It will detect about 1 part in 2,000 parts of urine. The great objection to this test in clinical work is that colour reactions are obtained with the excretion products of a number of drugs which are commonly employed in medicine (see above). It is wise always to check a positive finding with

Rothera's test by performing Gerhardt's test. If the latter is negative there is, as a rule, no need for alarm. If the ferric chloride test also is positive, a careful watch should be kept for clinical signs of acidosis, or, better, a determination of the alkali reserve should be made. A persistently positive ferric chloride test should be taken as a possible warning of impending acidosis, but such a finding of itself is often not of serious significance.

There is no simple test for  $\beta$  hydroxybutyric acid convenient for clinical use. If the dangers of ketosis be ascribed to acetoacetic acid as related above, the absence of such a test is of little moment in clinical work.

### The Detection of Acetone and Aceto-acetic Acid in Blood

Rothera's test can be applied directly to about 5 c.c. of non-hæmolyzed serum or plasma, and will detect a gross excess of the ketones, but it is not a good technique. It is better to precipitate the proteins first by tungstic acid and to test the protein-free filtrate. If the usual tungstic precipitation is made in whole blood, glutathione is liberated from the red cells, and therefore the filtrates invariably give a positive nitroprusside reaction. If, however, the proteins are precipitated without hæmolysis, as in Herbert and Bourne's method, using sodium sulphate and tungstic acid (p. 147), the filtrate does not contain significant amounts of glutathione, and Rothera's test may be applied to detect the ketones. The following technique is simple and requires only 0.2 c.c. of blood, which may be collected directly into a blood pipette from a puncture of the skin.

In a centrifuge tube place	{	3.6 c.c. of 3 per cent crystalline sodium sulphate
		0.2 c.c. of blood
		0.1 c.c. of 10 per cent sodium tungstate
		0.1 c.c. of $\frac{2}{3}$ N sulphuric acid

Mix rapidly, centrifuge at once and separate the supernatant fluid. To 2 c.c. of the supernatant fluid add 1 gm. of finely powdered ammonium sulphate, 0.1 c.c. of 5 per cent sodium nitroprusside prepared the same day, and 0.4 c.c. of concentrated ammonia (S.G. 0.88). Mix thoroughly and observe for fifteen minutes. Normal blood filtrates remain colourless, whilst those containing only a slight excess of the ketones slowly turn a pale purplish pink, the colour is very close to that of a similar thickness of 0.2161 per cent anhydrous cobaltous sulphate solution (0.1 Van den Bergh unit, cf. Chapter XII), in more marked ketonæmia a definite purple colour develops in a minute or less.

To deproteinise plasma or serum mix 1 c.c. with 8 c.c. of water, 0.5 c.c. of the sodium tungstate and 0.5 c.c. of the  $\frac{2}{3}$  N acid, giving a dilution of 1 in 10, or 3 c.c. of water may be used instead of 8 c.c. to give a dilution of 1 in 5.

Incidentally the stated proportions of supernatant fluid ammonium sulphate sodium nitroprusside and ammonia serve excellently to demonstrate the presence of glutathione in blood. Deproteinise by centrifuging a mixture of 1 c.c. of blood 7 c.c. of water 1 c.c. of the tungstate and 1 c.c. of the 2/3 N acid. Naturally the test is most conclusive if also the blood is shown to be free from any excess of ketones as above described.

### The Quantitative Estimation of the Acetone Bodies

The determination of the quantity of acetone bodies excreted daily in the urine, or of the concentration of these bodies in the blood, is often of the greatest interest in special investigations, but it can hardly be described, as yet, as a clinical procedure. The reader is, therefore, referred to larger works for the analytical procedures.

Normally the blood contains from 0 to 3 mgm. of total acetone bodies per 100 c.c. Acetone and aceto acetic acid account for 0 to 1 mgm. and  $\beta$  hydroxybutyric acid for 0 to 3 mgm. (as acetone).

Normally 20 to 50 mgm. of total acetone bodies (calculated as acetone) are excreted in the urine per diem. Of this 3 to 15 mgm. are in the form of acetone and aceto acetic acid, and 20 to 30 mgm. as  $\beta$  hydroxybutyric acid. In pathological states the ketonuria is not usually of much significance until more than 2 gm. of total acetone bodies are excreted in the twenty four hours.

### The Clinical Significance of Ketosis

Ketosis is due to the abnormal katabolism of fats which occurs when the supply or the metabolism of carbohydrates is inadequate. Ketonuria is thus a *sign* of abnormal fat metabolism, and is not a disease in itself. To a small extent the abnormal katabolism of certain amino acids derived from protein may also contribute to ketosis. In fact, ketosis results from a disturbance of the balance between the "ketogenic" and the "antiketogenic" substances. The acetone bodies are derived both exogenously, from the diet, and endogenously from the body. It has been calculated (Woodyatt) that each gramme of carbohydrate when metabolised yields 1 gm. of sugar ('antiketogenic'), but no fatty acids ('ketogenic'). Similarly each gramme of protein yields 0.58 gm. of "sugar" and 0.46 gm. of fatty acids or ketogenic amino acids expressed in terms of higher fatty acids, and each gramme of fat 0.1 gm. of "sugar" (glycerol  $\rightarrow$  glucose) and 0.9 gm. of fatty acids. When the ratio of ketogenic to antiketogenic substances exceeds a certain figure ketonuria results. Woodyatt, assuming a ratio of 1.5 to 1, calculated that ketonuria would not occur so long as the total fat metabolised did not exceed twice the amount of carbohydrate plus half the amount of protein, or, expressing the statement in a formula,  $F = 2C + \frac{1}{2}P$ , or, more accurately,  $F = 2C + 0.54P$ .

In diabetes there is a large excess of sugar in circulation, but that sugar is not properly or completely metabolised owing to a deficiency of endogenous insulin, ketosis, therefore, results. Diabetes is the condition *par excellence* in which ketosis occurs, but in clinical work ketosis is met with much more commonly in non diabetic than in diabetic conditions. In healthy adults



starvation will generally produce ketosis in twenty four hours. The actual period of starvation required before acetone bodies appear in the urine varies in different healthy individuals and depends, among other things, on the previous diet. Goldblatt notes that twenty hours sufficed if the previous diet had been low in carbohydrate, whereas thirty six hours might be required after an average mixed diet. In children the period is certainly much shorter, ketosis is often observed after twelve hours' starvation. In disease, also, the necessary period is often less than twenty four hours in adults, and in sick children deprivation of food may cause ketosis in six hours. In clinical work by far the commonest cause of ketosis is abstention from food, or reduction of the proportion of total available carbohydrate. Thus the preparation for an operation, of itself, often induces slight ketonuria before the anæsthetic is given. After operation the action of the anæsthetic and the added hours of fasting tend to increase the ketosis. In all conditions in which vomiting occurs, and these are legion, ketosis may result owing to the poor assimilation of food. Similarly in all states in which the patient is unconscious, semi conscious, or merely devoid of appetite, the balance between ketogenic and antiketogenic substances may easily be shifted sufficiently to give rise to ketosis. From this aspect alone it is easy to understand why ketosis is so common in clinical work, in most instances it is probably not necessary to postulate any causes other than disturbance of the physiological balance between ketogenic and antiketogenic substances. Anyhow, in pathological work such a cause should be the first to be considered and investigated, though there is evidence that in diseased states other factors are at work (*cf* section on alkalosis).

## ACIDOSIS AND ALKALOSIS

### Definition of Terms

- (1) Blood reaction  $\left\{ \begin{array}{l} \text{less alkaline than normal, below } pH\ 7.3 \\ \text{Acidæmia, or "uncompensated acidosis"} \\ \text{more alkaline than normal, above } pH\ 7.5 \\ \text{Alkalæmia, or "uncompensated alkalosis"} \end{array} \right.$

(2) Blood reaction normal, between  $pH\ 7.3$  and  $7.5$ , but alterations in the distribution of acid radicals relative to basic radicals of such a nature as to cause a tendency towards an acid reaction—acidosis, or "compensated acidosis". Similarly when the redistribution of acid relative to basic radicals indicates a tendency towards an alkaline reaction—alkalosis, or "compensated alkalosis".

The definition of acidosis as a decrease in the alkali reserve (plasma bicarbonate) should be abandoned, since a lowered alkali reserve may be present in alkalosis as above defined. Similarly the definition of alkalosis as an increased alkali reserve may be misleading. This point is referred to again later.

(3) Acetone bodies present—ketosis. This has already been discussed.

(4)  $\text{CO}_2$  content,  $\text{CO}_2$  capacity,  $\text{CO}_2$  tension and alkali reserve of blood

By  $\text{CO}_2$  content is meant the total  $\text{CO}_2$  that which is free, in physical solution, plus that which is combined, in chemical combination as bicarbonate, in the blood at the moment it is drawn, and before there can be any escape of  $\text{CO}_2$  into the air. To retard this escape of  $\text{CO}_2$  the blood is collected under paraffin, or by displacement over mercury. If it is desired to estimate the  $\text{CO}_2$  content of plasma, "true plasma" must be employed, i.e., plasma separated as soon as possible from the corpuscles by centrifuging under paraffin whole blood which has been collected under paraffin, so that from start to finish the plasma is prevented from losing gases. For details the reader is referred to Peters and Van Slyke's *Quantitative Clinical Chemistry*, vol II, 1932 p 54. The usual corrections are made for barometric pressure, temperature, and aqueous vapour pressure.

By  $\text{CO}_2$  capacity is meant the total  $\text{CO}_2$  (free and combined) measured dry and at N.T.P. which the blood will hold at a definite tension of  $\text{CO}_2$  usually 40 mm of Hg, or the tension of  $\text{CO}_2$  in normal alveolar air. If the determination is to be made on plasma, "true plasma" must first be separated by collecting and centrifuging whole blood under paraffin. After separation, the plasma may safely be left exposed to air, and later as required saturated with  $\text{CO}_2$  at a definite tension of  $\text{CO}_2$ . The term  $\text{CO}_2$  combining power, strictly speaking, should be applied to that portion of the  $\text{CO}_2$  capacity which is in chemical combination (cf Chapter XIX).

If whole blood when originally collected is exposed to air,  $\text{CO}_2$  will escape from the plasma into the air. As a result the plasma will become more alkaline.  $\text{HCl}$  and  $\text{H}_2\text{CO}_3$  will then pass from the corpuscles into the plasma, and there will be a further loss of  $\text{CO}_2$  into the air and a "chloride shift" (see later section). If the original  $\text{CO}_2$  tension of the whole blood as collected is known, it should theoretically be possible to reverse this process exactly by saturating the whole blood again with  $\text{CO}_2$  at the original  $\text{CO}_2$  tension, and from this "true plasma" could then be separated. But in practice the original  $\text{CO}_2$  tension of the blood is not known, and it is not safe, at any rate in pathological subjects, to assume that the blood  $\text{CO}_2$  tension is the same as the alveolar  $\text{CO}_2$  tension (which can easily be measured). In other words, the only safe procedure is always to separate "true plasma" immediately after collecting the blood under paraffin.

By  $\text{CO}_2$  tension or  $\text{CO}_2$  pressure is meant the pressure exerted by the  $\text{CO}_2$  in a mixture of gases, expressed in mm of Hg. Thus, suppose by analysis of such a gas mixture it is found that 5 per cent is  $\text{CO}_2$  the barometric pressure being 758 mm and the aqueous vapour pressure at the particular temperature being 13 mm. Then the  $\text{CO}_2$  tension of that gas mixture is  $\frac{5}{100} \times (758 - 13)$  mm Hg = 37.25 mm Hg.

The  $\text{CO}_2$  tension of blood cannot be measured directly because

$\text{CO}_2$  (and oxygen) is present not only in physical solution, but also in chemical combination. It is determined indirectly by determining the volumes of  $\text{CO}_2$  that are taken up by samples of the blood at different known pressures of  $\text{CO}_2$ . These results are plotted and constitute a  $\text{CO}_2$  dissociation curve or  $\text{CO}_2$  absorption curve (cf Christiansen, Douglas and Haldane). The pressure on this curve that corresponds to the volume of  $\text{CO}_2$  in the blood as actually determined gives the pressure of the gas in the blood. This particular point on the curve is often termed "the arterial point".

By *alkali reserve* or *plasma bicarbonate* is meant the volume of  $\text{CO}_2$  measured dry and at NTP which is expelled by acid from 100 volumes of "true" plasma which has been equilibrated with normal alveolar air (40 mm  $\text{CO}_2$ ) at a given temperature (usually room temperature), the volume of gas in physical solution being deducted (cf *Medical Research Council Special Report, No 72*).

### The Symbols $C_H$ and pH

The concentration of hydrogen in ionised form,  $C_H$ , may be expressed as so many grammes per litre of solution. Thus, in pure distilled water  $C_H = 1 \times 10^{-7}$  gm per litre. In other words pure distilled water contains 1 gm of ionised hydrogen in 10 million litres or 1 mgm in 10,000 litres, or 0.0001 mgm per litre, or 0.00001 mgm per 100 cc, or 0.01  $\gamma$  per 100 cc.<sup>1</sup>

Pure distilled water is, of course, absolutely neutral. It is very slightly dissociated, the number of hydrogen ions being equal to the number of hydroxyl ions and the product  $C_H \times C_{OH}$  being constant, viz

$$C_H \times C_{OH} = 1 \times 10^{-14}$$

$$C_H = C_{OH} = 1 \times 10^{-7} \text{ (at } 21^\circ \text{ C)}$$

(The constant increases rather rapidly with a rise in temperature). In all aqueous solutions the product  $C_H \times C_{OH}$  equals the same constant at the same temperature, so that in every aqueous solution there must be both hydrogen and hydroxyl ions. The quantity of ionised hydrogen in a solution is measured by the electrical conductivity. The electrical conductivity of distilled water is minute, but it has been measured directly. It has also been calculated by measuring the conductivity of a series of solutions of an acid of increasing dilution, plotting the results and continuing the curve through the point at which the number of hydrogen ions and hydroxyl ions are equal. From these observations and calculations it has been found that at  $21^\circ \text{ C}$  in a strictly neutral solution the concentration of ionised hydrogen is  $1 \times 10^{-7}$  gm per litre or 1 gm ion  $\times 10^{-7}$  per litre and likewise the concentration of hydroxyl ions is  $17 \times 10^{-7}$  gm per litre or 1 gm ion  $\times 10^{-7}$  per litre ( $H = 1$ ,  $OH = 17$ ). In a neutral solution there are an equal number of hydrogen ions and of hydroxyl ions and the concentration of hydrogen ions (at  $21^\circ \text{ C}$ ) is  $1 \times 10^{-7}$  gm per litre. In an acid

<sup>1</sup>  $\gamma = \frac{1}{1000}$  mgm, or 1 mgm = 1000  $\gamma$

solution there are more hydrogen ions than hydroxyl ions, and the concentration of hydrogen ions (at  $21^{\circ}\text{C}$ ) is greater than  $1 \times 10^{-7}$  gm per litre (*e.g.*,  $1 \times 10^{-6}$ ). In an alkaline solution there are less hydrogen ions than hydroxyl ions, and (at  $21^{\circ}\text{C}$ ) the concentration of hydrogen ions is less than  $1 \times 10^{-7}$  gm per litre, *e.g.*,  $1 \times 10^{-8}$ . In other words, the reaction of a solution depends on the relative number of hydrogen and hydroxyl ions. Even in an alkaline solution there are some hydrogen ions, though very few. The reaction, therefore, of any solution, whether it be acid, neutral, or alkaline, may be measured in terms of its hydrogen ion concentration.

Figures such as  $1 \times 10^{-7}$  are cumbersome, and Sorensen introduced the practice of expressing the results in terms of logarithms. He introduced the symbol *pH* to denote the "hydrogen ion exponent", *pH* is the common logarithm of the reciprocal of (H), or  $pH = -\log C_H$ .

Thus  $C_H = 1 \times 10^{-7}$  becomes  $pH = 7.0 = \text{neutral}$

and  $C_H = 1 \times 10^{-6}$       „       $pH = 6.0 = \text{acid}$

$C_H = 1 \times 10^{-8}$       „       $pH = 8.0 = \text{alkaline}$

This is simple, but the difficulty arises when the power to which 10 is raised is not a whole number. The following example should make the calculation clear. At  $18^{\circ}\text{C}$  an N/10 solution of HCl is ionised to the extent of 84 per cent. If it was completely ionised 1,000 c.c. of the solution would contain 0.1 gm. of ionised hydrogen. In fact, it contains only 0.084 gm. per litre

$$C_H \text{ therefore} = 0.084$$

$$\log C_H = \bar{2}.924 = -1.076$$

$$-\log C_H = -(-1.076)$$

$$\text{i.e. } pH = 1.076$$

The *pH* of an N/10 solution of HCl is 1.076 at  $18^{\circ}\text{C}$

Whilst there is little doubt that Sorensen's notation is of value in scientific work, it must be admitted that it has introduced a great deal of "mystery" in many circles. The average medical man is really puzzled by *pH*. Most of us are not accustomed to think in terms of logarithms. Though cumbersome, the results perhaps would be better understood if expressed in mgm. of ionised hydrogen per 100 c.c. of solution. Thus the *pH* of normal blood lies between 7.30 and 7.50, or its  $C_H$  lies between 0.000005 and 0.000032 mgm., or 0.005 and 0.0032  $\gamma$ ,<sup>1</sup> per 100 c.c. of blood. Admittedly the rows of 0s are clumsy, but it has been shown that a strictly neutral solution contains (at  $21^{\circ}\text{C}$ ) 0.00001 mgm., or 0.01  $\gamma$ , of ionised hydrogen per 100 c.c., and that an alkaline solution contains less than that. It is clear, therefore, from the above figures that normal blood is alkaline.

A table showing the relation of *pH* to  $C_H$  is given in the Appendix

<sup>1</sup> For explanation of  $\gamma$ , see footnote on previous page

### Buffers

Hydrochloric acid is a "strong" acid, and if a trace of it is added to distilled water it is completely ionised. There is, as a result, a marked increase in the concentration of hydrogen ions in the solution. Carbonic acid is a "weak" acid, and its solution in water is only slightly ionised, and there is only a small increase in the concentration of hydrogen ions. If hydrochloric acid is added to a solution of sodium bicarbonate (the bicarbonate being in excess), sodium chloride is formed and carbonic acid is liberated, but there is only a slight increase of the concentration of hydrogen ions, because carbonic acid is only slightly ionised. Sodium bicarbonate and other salts of weak acids are, therefore, termed "buffers". Other examples of buffers found in the body fluids are phosphates, plasma proteins and hæmoglobin.

### Indicators

Most indicators are substances which change colour with variations of the hydrogen ion concentration of a solution. These indicators themselves may be regarded as either weak acids or weak bases whose undissociated molecules have a different colour from that of the ions into which they dissociate. Each indicator changes colour over some particular  $pH$  range and an indicator is frequently selected for a given purpose because its colour change occurs over the particular range required (see Table of Indicators in Appendix). Thus in the range of  $pH$  exhibited by blood, phenol red is frequently chosen. Indicators vary in their sensitivity. Thus litmus is relatively insensitive, and is suitable when titrating strong acids with strong bases, but of little or no value when titrating weak acids or bases. Phenolphthalein is a sensitive indicator and is frequently selected when titrating organic acids, *e.g.*, when determining the normality of a NaOH solution by titration against a weighed amount of acid potassium phthalate (see Appendix).

Other indicators are substances which change colour at the completion of a chemical reaction, *i.e.*, when either the merest trace in excess of a substance A is added to a substance B, or just when the last trace of a substance C has reacted with a substance D. Thus starch is used as an indicator when titrating iodine with sodium thiosulphate. Iodine gives that sodium thiosulphate a colourless compound, but the end point, being a change in colour from the faintest yellow to colourless, would be very indefinite. Iodine combines with starch to give an intense blue. Starch is, therefore, added to the iodine solution when titrating with sodium thiosulphate so that just when the last trace of iodine has reacted with the added thio, there is a sudden change from blue to colourless.

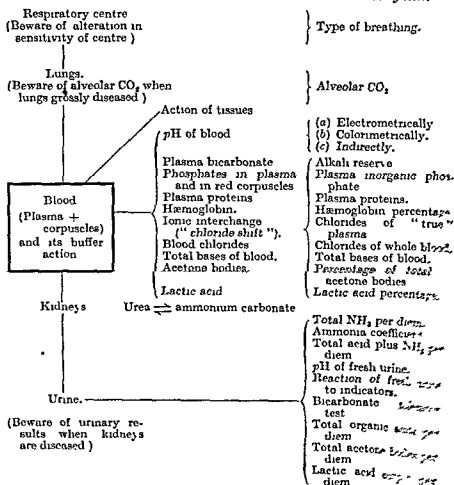
Indicators actually placed in the solution are termed "internal indicators" (*e.g.*, phenol red, phenolphthalein). Sometimes it is undesirable to put the indicator in the solution, it may often be

absorbed on to a piece of paper, to which a drop of the solution is added, or the paper may be dipped into, and rapidly withdrawn from, the solution (*e.g.*, litmus paper). At other times a drop of the indicator must be mixed with a drop of the solution, and the mixture then specially treated to bring about the colour change, *e.g.*, Gunzburg's reagent. In both these examples the indicator is termed an "external indicator." Some substances (*e.g.*, litmus) may be used either as an internal or as an external indicator.

## GENERAL DISCUSSION OF THE MECHANISMS CONTROLLING THE REACTION OF THE BLOOD AND BODY-FLUIDS

The reaction, the actual concentration of hydrogen ions, of the blood and body-fluids depends on a number of factors. The following diagram may assist in visualising the influence exerted by the different parts of the body in maintaining this reaction between the very narrow limits of pH 7.3 and 7.5 —

*Clinical observation or laboratory tests.*



The buffer action of the blood constitutes the first line of defence when abnormal acids, or abnormal amounts of acids or alkalies, are introduced into or formed in the body. In certain cases, of course, the lymph or body fluid immediately in contact with the acid or alkali will actually be the first warrior on the battlefield, but equilibrium between such body fluids and the blood circulating in the neighbourhood will rapidly ensue. The lungs under the control of the respiratory centre will then be called into play, making a coarse adjustment of the reaction of the blood. The kidneys finally will make a fine adjustment, and either by the complete excretion of all foreign matter will re-establish the original normal state, or by their continued action will bring about a new (dynamic) equilibrium. The action of the kidneys is relatively slow, and the adjustment by them a fine one. The action of the lungs and respiratory centre is rapid and relatively coarse. Actually these mechanisms come into play very nearly simultaneously, but it is convenient and roughly correct to think of them in the above order.

A more detailed discussion of the different mechanisms follows.

The Bases available for neutralisation of acids are ammonia, sodium, potassium, calcium and magnesium. Ammonia is present in minute quantities in the blood, but considerable amounts may be excreted in the urine in conditions of acidosis. This ammonia is formed from urea, and its seat of formation is possibly the kidney (Nash and Benedict). This mechanism is important in that it allows considerable quantities of acid to be excreted as ammonium salts, thereby conserving the other valuable bases. It follows that estimations of the daily output of ammonia would appear to be an important measure of the quantity of acids being excreted, and, therefore, of the risk of acidosis. In practice, however, the method is of limited value because (a) in renal disease the ability of the kidneys to excrete ammonia (or to convert urea into ammonia) is impaired, and (b) the quantity of ammonia excreted is not necessarily proportional to the risks of acidosis, because it is only one of the available mechanisms, and may be called into play in varying degree in different patients having a similar grade of acidosis. On the technical side it is essential to insure that ammonia is not formed by bacterial decomposition after the urine has been passed (see Chapter XVI).

Determination of the ammonia coefficient—the percentage of the total nitrogen which is ammonia nitrogen—is even less satisfactory owing to the very large variations that may occur in the excretion of the total nitrogen. The latter is influenced, above all, by the nitrogen intake, the amount of protein in the diet.

Estimations of the daily excretion of ammonia plus acid are of much more value, and are recommended by Fitz and Van Slyke as a measure of acidosis (see the table on p. 203).

The fixed bases, sodium, potassium, calcium and magnesium, are combined with the acid radicles in the blood and tissue fluids, but are available only to a limited extent for excretion, because the body fluids must be maintained at a fairly constant composition.

if life is to continue. Little information of value with regard to measuring acidosis can be obtained from an isolated estimation of any one of the fixed bases in blood or urine, but a determination of the total bases in blood and their distribution among the acid radicles is of real value, but too complicated as a routine clinical procedure. Analyses are of necessity practically limited to the blood and excretions, but all the body fluids and tissues play a greater or less part in the regulation of the acid base balance. Thus it has been shown that the administration of acids will finally lead to mobilisation of calcium salts from the bones. Possibly in renal infantilism the bony changes are mainly due to the prolonged acidosis resulting from the chronic and extensive renal lesions.

**The Distribution of the Acid and Basic Radicles in the Plasma may** be represented by the accompanying diagram (Fig. 42) taken from a paper by Gamble, Ross and Tisdall. The bases *B* are expressed in terms of cc N/10 per 100 cc of plasma, or milli equivalents per litre, and are the sum of the concentrations of Na, K, Ca and Mg. The acid radicles, similarly expressed, are the sum of  $\text{HCO}_3'$ ,  $\text{Cl}$ ,  $\text{HPO}_4'$ ,  $\text{SO}_4''$ , organic acids and protein. For the method of converting the results expressed in the more usual form of mgm per 100 cc, etc., into milli equivalents per litre, see Appendix, p 536. It will be seen that most of the base is combined with the strong acid, hydrochloric acid, and therefore is not immediately available for neutralisation of acid absorbed into or formed within the body, but some of it can be made available by ionic interchanges between plasma and corpuscles (see later under "chloride shift"). The minute proportion of base combined as sulphate is fixed and unavailable. Part of the base present as phosphate can be used. At the reaction of the plasma about 80 per cent is present as dibasic phosphate ( $\text{B}_2\text{HPO}_4$ ). The introduction of acid causes some of this to be converted into monobasic phosphate ( $\text{BH}_2\text{PO}_4$ ), base thereby being liberated to combine with the acid introduced. It will be seen, however, from the diagram that the base which could conceivably be rendered available from that combined with phosphates plus organic acids is small. The buffer action of the plasma proteins is illustrated

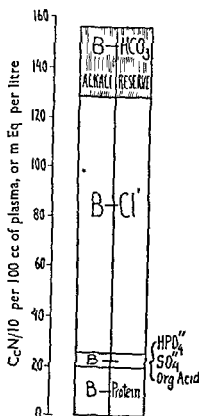


FIG. 42 The acid base composition of normal plasma (After Gamble, Ross and Tisdall.)



later in the section on the chloride shift, but it is doubtful whether the plasma proteins have a direct buffer action with regard to foreign acids absorbed into or formed within the body. It is clear, therefore, that the main "alkali reserve" of the plasma is in the form of  $\text{BHCO}_3$ , and this can be measured by a relatively simple technique (see end of chapter). The acid absorbed into or formed within the body reacts with the plasma bicarbonate ( $\text{BHCO}_3$ ), liberating  $\text{CO}_2$ , which is promptly excreted by the lungs. This is a rapid and very important mechanism preventing any change in the  $\text{pH}$  of the blood.

The Ratio of  $\text{H}_2\text{CO}_3$  to  $\text{BHCO}_3$ , in fact, as has been shown by Van Slyke, Henderson, Hasselbalch and others, is the most important factor controlling the  $\text{pH}$  of the blood. This ratio is 1 to 19 with a normal  $\text{pH}$  of 7.4. If the  $\text{H}_2\text{CO}_3$  content falls, the bicarbonate remaining constant, the blood will become more alkaline, the  $\text{pH}$  will rise. Conversely, if the bicarbonate content falls, the  $\text{H}_2\text{CO}_3$  remaining constant, the blood will become more acid, the  $\text{pH}$  will fall. So long as bicarbonate and  $\text{H}_2\text{CO}_3$  both alter proportionately and in the same direction, the  $\text{pH}$  will not alter. Moreover, an estimate of only one of the three variables, viz., bicarbonate content,  $\text{H}_2\text{CO}_3$  content, and  $\text{pH}$ , does not enable us to form a certain opinion as to the real condition of the patient. For this purpose, two of the three must be determined, and for this reason these methods are not commonly employed in clinical work. At the same time, an estimate of the total  $\text{CO}_2$ , or of the "alkali reserve" (bicarbonate) alone, is under certain conditions of value clinically. Thus if the "alkali reserve" is determined (see end of chapter) before and during administration of alkalis, it is easy to see whether there is a possible risk of alkalosis. Likewise in diabetic acidosis the estimation is valuable. Thus an "alkali reserve" of 25 to 40 vols  $\text{CO}_2$  per 100 c.c. of plasma is definitely an indication for treatment, though the acidosis may either be compensated, i.e., the low bicarbonate is accompanied by a corresponding fall in  $\text{H}_2\text{CO}_3$ , or uncompensated, i.e., the lowered bicarbonate is not accompanied by a corresponding fall in  $\text{H}_2\text{CO}_3$ . The latter is the more serious condition, and is accompanied by a fall of  $\text{pH}$ , i.e., an increase in the hydrogen ion concentration. Though in this region, viz., 25 to 40 vols  $\text{CO}_2$ , the estimation of the "alkali reserve" alone does not tell us whether the acidosis is compensated or not, when the result is 20 or under it may safely be assumed that the acidosis is not compensated and that the condition is very serious.

Following Peters and Van Slyke, the alterations in the  $\text{H}_2\text{CO}_3$  to  $\text{BHCO}_3$  ratio which occur clinically may be summarised as follows. The alterations in the acid base balance can be classified under two headings—*metabolic*, in which the primary change is either a deficit or an excess of alkali relative to acids other than carbonic and *respiratory*, in which the primary change is either a deficit or an excess of carbonic acid. In both groups there is at first no change in the  $\text{pH}$  of the blood, the acidosis or alkalosis is

"compensated" as explained above, later the  $pH$  may fall or rise respectively, the condition then being "uncompensated"

**Metabolic Primary Alkali Deficit, or Metabolic Acidosis.** The deficit of alkali is due to the fixation of base by acetoacetic or  $\beta$  hydroxybutyric acid as in diabetes mellitus, or by retained phosphoric acid as in advanced chronic nephritis, or by the administration of acidic substances. These will be considered in more detail later in discussing their clinical significance

$BHCO_3$  falls  $H_2CO_3$  falls at first correspondingly, but later not so fast, therefore the blood  $pH$  at first is normal, but in the terminal stages it falls. The alveolar  $CO_2$  falls. Clinically there is hyperpnoea (excretion of  $CO_2$  by the lungs), diuresis (excretion of Na salts and corresponding amounts of water by the kidneys) and dehydration. In the urine there is an accelerated excretion of water, of acids and of ammonia, except that in kidney disease the ammonia excretion fails

**Metabolic Primary Alkali Excess, or Metabolic Alkalosis.** This may be due to the retention of alkali as after the administration of alkalis (particularly sodium bicarbonate), or to the loss of acid, e.g., loss of  $HCl$  in severe vomiting

$BHCO_3$  rises  $H_2CO_3$  rises at first correspondingly, but later not in proportion, therefore the blood  $pH$  at first is normal, but later rises. The alveolar  $CO_2$  rises. Clinically there is excessive muscular irritability, leading eventually to tetany when the blood  $pH$  reaches about 7.6. The urine becomes alkaline, bicarbonate is excreted, but the ammonia decreases and may disappear, there is moderate diuresis, and may be ketonuria

**Respiratory Primary  $CO_2$  Excess (Respiratory Acidosis)** This occurs when air rich in  $CO_2$  is breathed (industrial poisoning), or when  $CO_2$  cannot be properly excreted owing to diseases of the lungs (pneumonia, pulmonary oedema, emphysema etc.), to gross obstruction of the air passages, or to deadening of the respiratory centre as in morphine poisoning

$H_2CO_3$  rises  $BHCO_3$  rises at first in parallel, but finally less quickly, so that the blood  $pH$  at first is normal, but later falls. The alveolar  $CO_2$  rises. Clinically this group is not of much importance, because, apart from  $CO_2$  industrial poisoning, death from anoxia must occur before  $CO_2$  accumulation becomes dangerous. The breathing is increased, in depth more than in frequency. There is increased urinary ammonia, titratable acidity, and acid phosphates.

**Respiratory Primary  $CO_2$  Deficit (Respiratory Alkalosis).** This is caused by overbreathing which "washes out"  $CO_2$ . The overbreathing may be due to oxygen want, as in heart disease, gross anæmia,  $CO$  poisoning and at high altitudes, or to an attempt to reduce hyperpyrexia, as in some fevers, in exposure to hot dry air or prolonged hot baths, or to irritation of the respiratory centre, as in some cases of encephalitis, or it may be voluntary or hysterical

$H_2CO_3$  falls  $BHCO_3$  falls at first in parallel, later less quickly, so that the blood  $pH$  at first is normal, but later rises. The alveolar  $CO_2$  falls. Clinically there is, of course, hyperpnoea, and, in those

cases in which the blood pH exceeds about 7.6, tetany. The urine becomes alkaline, contains bicarbonate (though less than in metabolic alkalosis), but little or no ammonia. There is diuresis and may be slight ketonuria.

Before attempting to discuss the above from the clinical point of view, there are two further mechanisms of importance in maintaining the reaction of the blood at a constant level, viz., the oxyhæmoglobin-hæmoglobin change and the chloride shift.

The Oxyhæmoglobin-hæmoglobin Change is of importance in preventing changes in reaction which would otherwise occur when  $\text{CO}_2$  is added to blood in its passage through the tissues, and when  $\text{CO}_2$  is lost from the blood during its passage through the lungs. At the reaction of the body both oxyhæmoglobin and hæmoglobin behave as weak acids, but of the two bodies oxyhæmoglobin is more strongly acid. In the lungs  $\text{CO}_2$  leaves the blood and hæmoglobin is changed to oxyhæmoglobin. The loss of acid in the form of  $\text{CO}_2$  is balanced by the increase of acid owing to the formation of oxyhæmoglobin.

Conversely, in the tissues the increase in acid owing to addition of  $\text{CO}_2$  to the blood is balanced by the loss of acid due to the reduction of oxyhæmoglobin to hæmoglobin. This mechanism maintains an approximately constant pH in the blood whether it be arterial or venous, but it cannot be of much importance in conditions of acidosis due to such acids as acetic or phosphoric which are not excreted by the lungs, seeing that of necessity hæmoglobin is changed to oxyhæmoglobin in the lungs.

The chloride shift is another mechanism tending to prevent a change of pH when the blood takes up  $\text{CO}_2$  from the tissues or gives up  $\text{CO}_2$  in the lungs. In the tissues, with increase of  $\text{CO}_2$  tension, there is a migration of Cl ions from plasma to corpuscles, thus leaving more base in the plasma to combine with the  $\text{CO}_2$ . In the corpuscles the incoming Cl ion is buffered in part by the phosphates, and in part by the hæmoglobin. In the lungs the reverse process occurs. The changes that occur may be represented by the following scheme (Van Slyke) —

Plasma	Red Cell Wall	Red Cell
$\text{H}_2\text{CO}_3 + \text{NaCl} \rightleftharpoons \text{NaHCO}_3 + \text{HCl}$	$\leftarrow \text{HCl} \rightarrow$	$\begin{cases} \text{HCl} + \text{K}_2\text{HPO}_4 \rightleftharpoons \text{KH}_2\text{PO}_4 + \text{KCl} \\ \text{HCl} + \text{K HbO} \rightleftharpoons \text{H HbO} + \text{KCl} \\ \text{HCl} + \text{K Hb} \rightleftharpoons \text{H Hb} + \text{KCl} \end{cases}$
$\text{H}_2\text{CO}_3 + \text{Na protein} \rightleftharpoons \text{NaHCO}_3 + \text{H protein}$	$\leftarrow \text{H CO}_3 \rightarrow$	$\begin{cases} \text{H}_2\text{CO}_3 + \text{K}_2\text{HPO}_4 \rightleftharpoons \text{KHCO}_3 + \text{KH PO}_4 \\ \text{H CO}_3 + \text{K HbO} \rightleftharpoons \text{KHCO}_3 + \text{H HbO} \\ \text{H CO}_3 + \text{K Hb} \rightleftharpoons \text{KHCO}_3 + \text{H Hb} \end{cases}$
$\text{O}_2$ —————	$\leftarrow \text{O}_2 \rightarrow$	$2\text{H HbO} \rightleftharpoons 2\text{H Hb} + \text{O}_2$

The following table summarises the findings in arterial and venous blood respectively in a healthy individual —

(From Hawk and Bergem's *Practical Physiological Chemistry*)

	ARTERIAL BLOOD			VENOUS BLOOD		
	Whole Blood	Plasma	Cells	Whole Blood	Plasma	Cells
pH	—	7.45	7.31	—	7.42	7.30
Free CO <sub>2</sub> vol per cent of whole blood	2.4	1.6	0.8	2.7	1.8	0.9
Combined CO <sub>2</sub> vol per cent of whole blood	45.8	34.0	11.8	50.4	36.8	13.6
NaCl mgm per 100 cc whole blood	468	349	119	463	342	126
Combined O <sub>2</sub> vol per cent of whole blood	19.3	—	—	13.0	—	—
Saturation of Hb per cent	96.0	—	—	60.0	—	—

This chloride shift also occurs to a greater or less extent in any acidosis or alkalosis and is most marked in acidemia and alkalemia.

From the above table, whole blood chlorides would seem to give either no information or not as much as the plasma chlorides. The table, however, applies to a normal individual (whole blood chlorides unaltered). In pathological conditions, the chloride shift may be exaggerated (cf intestinal obstruction, p 194) in which case there may be also an alteration in the whole blood chlorides. In fact, in pathological studies, the chloride shift only becomes significant when whole blood chlorides are altered. Therefore, for clinical work it is recommended that estimations be made on whole blood. This is fortunate in view of the fact that a chloride shift from corpuscles to plasma occurs *in vitro* unless escape of blood gases is prevented, "true plasma" (see p 179) must be employed for estimating plasma chlorides.

## THE CLINICAL APPLICATION OF THE ABOVE DATA

### Acidosis and Acidemia

In clinical medicine acidosis may be broadly considered under five headings, due to —

Excessive formation of aceto acetic and  $\beta$  hydroxybutyric acids

Excessive formation of other organic acids

Failure of phosphate excretion

Administration of acidic substances

Failure to excrete H<sub>2</sub>CO<sub>3</sub> properly

**Acidosis due to Excessive Formation of Ketone Bodies** Diabetes mellitus is the classical example, and acidosis has been extensively studied in this disease. In the early stages of ketosis there is no alteration in pH, and not even any appreciable fall in the alkali reserve. The ketone bodies are in a low concentration in the blood and are rapidly excreted in the urine, partly as sodium or ammonium salts and partly free. As the ketosis increases, a significant portion of the base of the blood bicarbonate is fixed by the aceto acetic

and  $\beta$  hydroxybutyric acids wherefore the alkali reserve falls. But the free  $\text{CO}_2$  ( $\text{H}_2\text{CO}_3$ ) falls in parallel and no change in pH occurs. In other words, there is a compensated acidosis. Only in the terminal stages of the disease does this compensatory mechanism fail, and a fall in pH, a true acidæmia, results. The body tries to compensate for the fall in the alkali reserve even in these terminal stages by rapid breathing, hence the typical air hunger in diabetic coma. Treatment by alkali is not indicated until the terminal stages are approached, because there is no serious loss of alkali from the body, the alkali being simply fixed by the foreign acids. Insulin (plus abundance of fluids, warmth, etc.) is indicated to prevent the formation of ketone bodies, and when the ketone bodies already in circulation are eliminated, the alkali reserve returns to normal. In diabetic coma there has already been a loss of alkali, and sodium bicarbonate may be given with advantage for a short time, e.g., twenty four to forty eight hours, together with insulin etc. But if alkali treatment is prolonged there is a risk of causing alkalosis, and it has even been suggested that in that case the formation of ketone bodies may recur as a means of fixing some of the excess alkali.

It has already been pointed out in the first part of this chapter that ketosis is much commoner in non diabetic conditions than in diabetes. Thus starvation, whether absolute or merely relative (e.g. in vomiting, severe anorexia, etc.), is a common cause. Similarly in the pernicious vomiting of pregnancy and in the cyclical vomiting of childhood, ketosis may be marked, but such evidence as is at present available leads to the general conclusion that in these non diabetic conditions there is often no acidosis at all, or when there is acidosis it is compensated. Rarely, if ever, is there uncompensated acidosis or acidæmia, and, therefore, it is only exceptionally that alkali treatment is justifiable.

**Acidosis due to Excessive Formation of other Organic Acids**  
Clausen has found in children that an acidosis due to lactic acid may occur in cases of circulatory failure, especially in cardiac decompensation and anhydræmia. Acidosis is not present in all cases of anhydræmia. Ketosis is absent or relatively slight in lactic acid acidosis. This type of acidosis is adequately relieved by treatment of the underlying circulatory condition and alkalis are not indicated and if given may lead to alkalosis.

The same author has shown in children that large quantities of organic acids are excreted in the urine after the crisis in pneumonia. The larger part of these organic acids is insoluble in ether.

**Acidosis due to Failure of Phosphate Excretion** In gross renal inefficiency of the azotæmic type (advanced chronic interstitial nephritis, severe renal infantism, late stage of congenital cystic kidney disease, etc.) the kidneys are unable to excrete phosphates. The plasmic inorganic phosphate is increased (cf Chapter V) the alkali reserve and the serum calcium are lowered. There is a true acidosis, which in the earlier stages is compensated, i.e., it is accompanied by a corresponding fall in the free  $\text{CO}_2$  of the blood (alveolar

$\text{CO}_2$  low), but in the later stages is uncompensated, there being a fall in the  $\text{pH}$  of the blood and an acidæmia. The quantity of ammonia excreted in the urine is decreased, an observation best explained by the hypothesis of Nash and Benedict that ammonia is normally formed from urea by the kidney. With gross damage to the kidneys it would be expected, on this hypothesis, that the ammonia excretion would fall. The patients in this group who vomit considerably may not show as big a drop in the alkali reserve as would be expected from the degree of phosphate retention. This may be explained by the loss of  $\text{HCl}$  in the vomit, which by itself would tend to lower the plasma chloride and to increase the alkali reserve (*cf* p 194). From the above findings it is a natural suggestion that administration of alkalies and of calcium lactate would be valuable, and such remedies may possibly prolong life for a short while, but owing to the fact that it is impossible to repair the renal damage no treatment is of much avail.

**Acidosis due to Administration of Acidic Substances** This type of acidosis is not common in clinical work. Theoretically it could arise from excessive administration of any acid or acid salt. Practically it is most likely to result from breathing air containing a high proportion of  $\text{CO}_2$  or after the employment of excessive doses of calcium chloride or ammonium chloride. Mixtures of oxygen and carbon dioxide with nitrous oxide are used for producing anaesthesia, but the proportion of  $\text{CO}_2$  is carefully regulated, and the writer is not aware of any clinical acidosis arising therefrom. Large percentages of  $\text{CO}_2$  may be encountered in sewers, mines, and so on, but if clinical signs of distress arise they are usually due more to other causes (*e.g.*,  $\text{CO}$  poisoning), and the  $\text{CO}_2$  acidosis, if it exists, is of secondary importance. Calcium chloride in the intestine or body fluids (intravenous injection) tends to dissociate, the calcium being bound as phosphate, as soaps, or as proteinate, and the chlorine reacting with  $\text{BHCO}_3$  and thereby fixing base and liberating  $\text{CO}_2$  which is excreted by the lungs. Ammonium chloride similarly causes an acidosis, the ammonia combining with  $\text{H}_2\text{CO}_3$  and being converted into urea, and the chlorine reacting with  $\text{BHCO}_3$ . At first a compensated acidosis results, but it is possible by continuous administration of large doses of these two drugs to cause an actual acidæmia, and it has been shown that calcium salts may actually be mobilised from the bones as a result.

In the treatment of pernicious anaemia hydrochloric acid is frequently given, but it is difficult, if not impossible, to give sufficient quantities to cause acidosis. The same applies to the therapeutic use of acid sodium phosphate, phosphoric acid, etc., in urinary complaints, and so on, but  $\text{NaH}_2\text{PO}_4$  may increase the acidosis seriously if renal damage is severe.

The organic acids, such as citric, tartaric, etc., are oxidised in the body to carbonic acid, which is quickly eliminated by the lungs. They, therefore, do not cause acidosis. In fact, the alkali salts of these acids, *e.g.*, sodium citrate are valuable alkalies owing to their conversion into bicarbonates.

**Acidosis due to Defective Elimination of Carbonic Acid** This occurs when the lungs are grossly damaged, as in pneumonia, severe bronchitis, pulmonary œdema, and emphysema, or grossly obstructed, as in laryngeal obstruction (*cf* Negus)

In many cases the increase in  $\text{H}_2\text{CO}_3$  is compensated by a corresponding increase in  $\text{BHCO}_3$ , but in others the acidosis is not compensated and a fall in pH (acidæmia) occurs

Owing to the pathological changes in the walls of the pulmonary alveoli, the alveolar  $\text{CO}_2$  tension may actually be a good deal lower than the blood  $\text{CO}_2$  tension

In decompensated heart disease, not associated with gross pathological changes in the lungs, there is not an acidosis, but when complicated by pulmonary lesions there may be (*cf* Fraser)

The methods employed, and the calculations necessary, in studies of the respiratory metabolism and acid base balance in cases of cardiac and pulmonary disease are too complicated to be applied as routine tests, though they are of the greatest value in the scientific investigation of the cause of various clinical signs and of the rational basis for various lines of treatment

### Alkalosis and Alkalæmia

In clinical work alkalosis may be grouped under three headings

Administration of alkalis

Loss of  $\text{HCl}$  or  $\text{NaCl}$  by vomiting

Loss of carbonic acid

**Alkalosis due to Administration of Alkalis** Treatment by alkalis has long been widely used in medicine, but only comparatively recently has it been shown that the alkali can do harm in certain instances. Indeed, in many of these it is not so much the alkali actually given as the fact that, in addition, other lines of treatment simultaneously restore the normal alkali reserve of the body. Thus at one time large quantities of alkali were prescribed over long periods in diabetes mellitus. This was originally done with the idea of neutralising the aceto acetic and  $\beta$  hydroxybutyric acids formed in this disease, but in the absence of quantitative estimations of these bodies and with the necessity of gauging the dose of alkali by rough tests, such as the reaction of the urine to litmus an alkalosis and even an alkalæmia must, and, in fact, did occasionally result. That was in the days when alkali was combined with dietetic treatment. Now that insulin is also available and rapidly prevents the formation of the foreign acids the dangers of alkalosis from coincident alkali treatment are even greater. As mentioned previously, in diabetes the acidosis is compensated until the terminal stages, and even in coma it is advisable to give alkali for a short period only, owing to the rapid action of insulin in restoring the alkali reserve.

Alkalosis has also been reported in the alkaline treatment of gastric ulcer, of pyæmia, of tetany, and so on. In some forms of tetany there is already an alkalosis (*cf* p 188), and so alkaline

treatment is contra indicated. In nephritis there is a risk of causing cedema as well as alkalosis if alkali is pushed.

Palmer and Van Slyke have shown that in normal individuals when the alkali reserve rises to a figure of about 71 volumes of  $\text{CO}_2$  per 100 c.c. of plasma, the reaction of the urine approximates to that of the blood and becomes faintly alkaline, and when the alkali reserve rises still higher the urine becomes definitely alkaline. In most of the pathological cases studied, however (mainly diabetes and nephritis), the urine did not become alkaline until a higher plasma bicarbonate had been reached. In other words, in disease it is not always safe to be guided by the reaction of the (fresh) urine to litmus. It is advisable to control the amount of alkali given by estimations of the alkali reserve (see end of chapter). The same authors have shown that from a preliminary estimation the dose of sodium bicarbonate necessary to restore the alkali reserve to a normal level may be calculated thus —

1 gm. of  $\text{NaHCO}_3$  yields 267 c.c. of  $\text{CO}_2$  at  $0^\circ$  and 760 mm

1 kgm. of body weight contains 700 c.c. of water approximately

Let the body weight in kgm. be  $W$ . The body fluid will then be  $700 \times W$  c.c. For each 1 gm. of  $\text{NaHCO}_3$  given,

$700 \times W$  c.c. of body fluid will contain an extra 267 c.c. of  $\text{CO}_2$

100 c.c.                    "                    "                    "                    "                     $\frac{267}{7 \times W}$  "

or  $\frac{38}{W}$  c.c.  $\text{CO}_2$

Conversely, suppose it is desired to raise the body fluid  $\text{CO}_2$ -content by  $x$  c.c. of  $\text{CO}_2$  per 100 c.c.

Since a rise of  $\frac{38}{W}$  c.c. of  $\text{CO}_2$  is caused by 1 gm. of  $\text{NaHCO}_3$

$\therefore$  " 1 " " " "  $\frac{W}{38}$  "

$\therefore$  "  $x$  " " " "  $\frac{x \times W}{38}$  "

For example, if the original plasma bicarbonate (and it is assumed the original body fluid bicarbonate) is 60 c.c. of  $\text{CO}_2$  per 100 c.c., an increase of 11 c.c. of  $\text{CO}_2$  per 100 c.c. is desired, i.e.,  $x = 11$

The bicarbonate required is  $\frac{11 \times W}{38}$  gm., which for an indi-

vidual of 57 kgm. (9 stone) would be  $\frac{11 \times 57}{38} = 16.5$  gm.

Palmer and Van Slyke found in normal individuals that the observed rise in  $\text{CO}_2$  was very nearly that which would be expected by calculation, but in disease the observed was often less than the calculated rise. In other words, the above method of calculation will prevent the giving of too much bicarbonate in disease.

In practice, however, the necessary estimations of the alkali



reserve (plasma bicarbonate) and the above calculations are often impossible. In that case reliance must be placed on testing the reaction of the fresh urine with litmus, remembering that in certain cases in which large doses of alkali are necessary, there is a possibility of alkalosis, although the urine may be scarcely alkaline.

There is evidence that large doses of bicarbonate may be the direct cause of ketosis, and it has been suggested that the formation of acetoacetic and  $\beta$  hydroxybutyric acids is a mechanism for counteracting the alkalosis. This completely upsets the old view that the presence of ketone bodies points to an acidosis and illustrates once more the absolute necessity of differentiating between ketosis and acidosis.

**Alkalosis due to Loss of HCl or NaCl by Vomiting** In high intestinal obstruction (pyloric obstruction in adults, congenital pyloric obstruction of infants, obstruction of duodenum or of upper jejunum) there is a rise in the plasma bicarbonate and a fall in blood chloride. In fact, in all examples of excessive vomiting there is a tendency to the same state of affairs, though the picture may be modified by other influences. Thus in uræmic vomiting the acidosis due to retention of phosphates more than counterbalances the alkalosis due to vomiting (*cf* p 191).

The most obvious explanation of these findings is the loss of HCl in the vomit. The hydrochloric acid of the gastric secretion is formed from the sodium chloride of the blood. The sodium combines with carbonic acid to form bicarbonate, which is carried away in the blood from the stomach. Later in the cycle of digestion sodium bicarbonate is passed into the intestine in the pancreatic and intestinal secretions. In the intestine the HCl from the stomach reacts with this bicarbonate to reform sodium chloride, which is absorbed and so the plasma chloride is readjusted. If, however, HCl is lost by vomiting this cycle is broken, and the blood is left rich in bicarbonate and poor in chloride. There are, however, several difficulties in accepting this hypothesis. Thus the vomit may contain no free HCl, and it has been stated that ligation of the œsophagus causes similar changes. Moreover, in obstruction of the lower part of the duodenum or upper part of the jejunum, it would be expected that not only HCl from the stomach, but also bicarbonate from the pancreatic secretion would be vomited. An alternative hypothesis is that the blood changes are due to loss of mineral chloride (mainly NaCl) in the vomit (*cf* Hartmann and Smyth). Base is mobilised from the tissues and combined with carbonic acid, thus keeping up the bicarbonate content and the isotonicity of the blood. When this supply of base begins to fail, non protein nitrogen is retained, thus accounting for the raised urea and non protein nitrogen observed in cases of severe vomiting. As mentioned in Chapter V, one of the functions of the kidney is the regulation of the osmotic pressure of the blood, and when the kidney is unable to carry out this function by not excreting chlorides, it is suggested that it does so by not excreting as much non protein nitrogen as normally. This hypothesis would account for the fall

in blood chloride in vomiting due to intestinal obstruction at any level, or to general peritonitis, and is supported by the experimental work of Haden and Orr. When sodium chloride is given in these conditions the blood chloride, bicarbonate and non protein nitrogen rapidly return to normal, and the toxic symptoms are largely or completely relieved, provided that the intestine is not gangrenous and that the obstruction is removed. When the latter conditions do not hold, chloride administration corrects the blood chemical disturbances, but does not, of course, prevent the absorption of toxins from the damaged mucous membrane. The great clinical importance of chloride treatment in intestinal obstruction and peritonitis does not appear to be widely appreciated in this country.

Anrep and Cunnan have shown that the concentration of lactic acid in the blood rises when the alkalinity of the blood is increased, and suggest that this is another mechanism for preventing changes in the reaction of the blood to the alkaline side.

**Alkalosis due to Loss of Carbonic Acid** This occurs in hyperpnœa, whether due to oxygen want (e.g., at high altitudes, or in heart failure not associated with gross pulmonary lesions), or voluntary, or due to prolonged hot baths, and may be regarded as a simple washing out of  $\text{CO}_2$  by the excessive breathing. If the plasma bicarbonate falls proportionately there is no change in the pH of the blood and the alkalosis is compensated. If the fall in bicarbonate does not keep pace with the fall in  $\text{CO}_2$  tension, there is uncompensated alkalosis, or alkalemia, and the pH of the blood rises. In thermic (hot bath) fever the pulmonary ventilation may be enormously increased and ketosis may result (cf. Bazett and Haldane). This ketosis is probably a compensatory mechanism, and provides yet another example of the association of ketosis with alkalosis. Both  $\text{CO}_2$  tension and plasma bicarbonate are low—an instance of the misleading result of labelling a lowered alkali reserve (plasma bicarbonate) an "acidosis." In cardiac failure some of the patients exhibit a compensated, others an uncompensated,  $\text{CO}_2$  deficit (compensated alkalosis, and alkalemia respectively), presumably the result of increased pulmonary ventilation due to anoxæmia, so long as there is no gross pulmonary lesion. When the heart failure is complicated by severe pulmonary disease (extensive pulmonary œdema, pneumonia, etc.), there may be an actual acidosis due to the inability of the pulmonary alveoli to allow  $\text{CO}_2$  to pass through properly. Owing to the opposing action of cardiac failure and pulmonary disease on the blood reaction, the nett result, in some patients may be an acid base balance within normal limits.

Koehler has shown that in some fevers (influenza) an alkalosis may be present, probably due to the increased pulmonary ventilation. The alveolar  $\text{CO}_2$  is low, and this at first sight might be regarded as evidence of "acidosis," whereas actually it is in this case evidence of a lowered blood  $\text{CO}_2$  tension, which may be accompanied by a proportionately lowered plasma bicarbonate (compensated alkalosis), or by a plasma bicarbonate which has not fallen in proportion (alkalemia). This alkalosis in fevers may be accompanied by a

*ketosis*—another example of this association. The *ketosis* may be due to a compensatory mechanism to reduce the *alkalosis*, or to *anorexia* causing a partial carbohydrate deficiency, or to both. The *tetany* which occasionally occurs in acute fevers may be ascribed to *alkalosis*.

### CONCLUDING REMARKS ON THE CLINICAL VALUE OF DETERMINATIONS OF THE ACID-BASE BALANCE

It will be seen from the above discussion that determinations of the acid base balance in pathological states must often be a comparatively complicated procedure, and, therefore, seldom applicable to clinical cases in ordinary practice. When dealing with diseases that have been thoroughly studied, it is justifiable from previous work to assume that an *acidosis* or *alkalosis*, as the case may be, is present, and to use an incomplete technique to measure the *acidosis* or *alkalosis*. For example, in *untreated diabetes mellitus* with *acetone bodies* in the urine, it may be assumed that there is an *acidosis*, and help in treatment can be obtained by measuring the *alkali reserve* (*plasma bicarbonate*). Again, in patients given large doses of sodium bicarbonate, the same method can be used as a guide to subsequent dosage with alkali. But in dealing with conditions less well studied, an incomplete technique (e.g., a measurement of the *alkali reserve* alone) may be more misleading than no laboratory tests at all.

The old clinical view that *acidosis* and *ketosis* are synonymous can no longer be maintained, but from the essentially practical point of view *acidosis* due to the *ketone bodies* is still the most important clinically. *Acidosis* in chronic kidney disease is usually of minor importance because generally the renal damage cannot be repaired, in acute nephritis and in acute obstruction of the urinary passages, however, attention to the acid base balance of the blood may be of therapeutic value. The practical man would point out that the dangers of administration of acids or of alkalis are obvious, but the newer methods have put the risks on a quantitative basis. The *alkalosis* of severe vomiting again is more of academic than of practical interest because, if the cause of the vomiting is successfully treated, the *alkalosis* is soon corrected by the natural mechanisms. Finally, *respiratory acidosis* is admittedly (p. 187) of secondary importance to the *anoxia*, and *respiratory alkalosis*, too, is of little importance clinically, for when the oxygen want, *hyperpyrexia*, etc. (p. 187), can be successfully treated the *alkalosis* disappears.

The practical man, therefore still tends to maintain that for him *ketosis* is almost the only condition in which he need bother about "acid base balances," but there is a real danger of his becoming unduly alarmed by *ketonuria*, which has been the cause of much unnecessary worry, and possibly on occasion has led to bad treatment. In *diabetes mellitus* the issue is generally clear. Slight *ketosis* (a positive *Rothera's* test, but a negative *Gerhardt's* test)

rarely matters. Moderate or marked ketosis (a positive or strongly positive ferric chloride reaction) is undesirable, but it may or may not be the accompaniment of a serious state of acidosis. In non-diabetic ketosis there is seldom any need to worry at all about the finding of acetone bodies in the urine. Such ketosis may be associated with compensated acidosis, compensated alkalosis, or rarely with acidæmia or alkalmia, but in most cases without elaborate investigation it is impossible to form an opinion as to the reaction of the blood, or as to the risk of the blood pH shifting into dangerous zones.

Unfortunately determinations of blood pH are technically comparatively difficult, but fortunately this does not matter much in ordinary clinical work, because the pH is not altered significantly until the patient is very gravely ill or moribund, so delicate and so elaborate are the various mechanisms for keeping it practically constant. (Normally the range is pH 7.3 to 7.5, and the most extreme variations reported in disease almost all lie between pH 7.0 and 7.8.) The idea that if only there was a simple way of measuring blood pH much valuable information in a whole host of diseases could be obtained, is therefore, obviously wrong. What should be valuable in clinical work is a simple method of measuring the *tendency* to an acid state (acidosis) or the *tendency* to an alkaline state (alkalosis) of the blood, but in view of the many interdependent factors outlined above, this clinical ideal of a simple test seems unlikely ever to be realised.

### TECHNICAL

#### Note on Total CO<sub>2</sub> Content of Venous True Plasma or Serum

Van Slyke (see Peters and Van Slyke, vol. 1, p. 935) has largely discarded the determination of the alkali reserve in favour of the total CO<sub>2</sub> content of venous plasma or serum separated without loss of gases from blood which has been collected without loss of gases and without constriction of the vein. The writer feels that the technique (see Peters and Van Slyke, vol. 1) necessary for the collection and centrifugation of the blood would commonly be too difficult in everyday clinical work, and unless the proper technique is used serious errors might result. In patients it is often difficult to obtain venous blood without some degree of stasis. At present, therefore, it is the writer's practice to determine the alkali reserve in the old way, as described in the next section.

In health the total CO<sub>2</sub> content of venous true plasma, collected without stasis, varies from 55 to 74 c.c. of CO<sub>2</sub> per 100 c.c. (Peters and Van Slyke, vol. 1, p. 941), which is very similar to the normal range for the alkali reserve (53 to 77 c.c. of CO<sub>2</sub> per 100 c.c.)

#### Determination of Alkali Reserve or Plasma Bicarbonate

**Principle** "True" plasma from oxalated blood collected under liquid paraffin is shaken with air whose carbon dioxide tension approximates to that of normal arterial blood (usually normal alveolar air), causing the plasma to combine with as much carbon

dioxide as it is able to hold under normal tension. A known quantity of the plasma is then acidified within a suitable apparatus and its carbon dioxide liberated by the production of a known partial vacuum. The volume of the liberated carbon dioxide is then measured at atmospheric pressure and calculated for 100 c.c. of plasma.

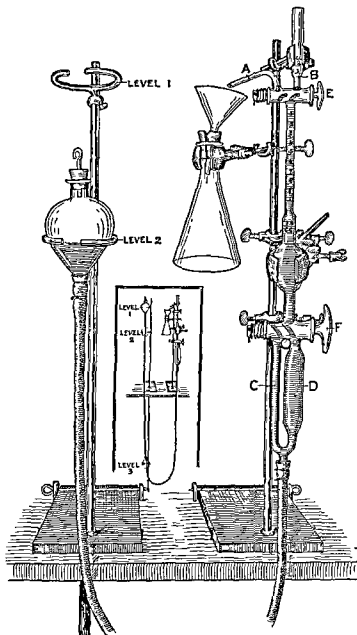


FIG 43. Van Slyke's carbon dioxide apparatus. Level 1. Surface of mercury in levelling-tube just above horizontal plane through E. Level 2. Just below plane through 25 c.c. mark. Level 3. About 2 ft. 10 in. below level 2, i.e., low enough to bring the mercury in the evacuator about half way down C and D, when E is closed.

**Apparatus** The Van Slyke carbon dioxide apparatus (Fig 43) is supported in a powerful screw clamp, the jaws of which are lined with rubber pads, and additional support in the form of an iron rod is arranged to project through the two limbs C and D under stop cock F to support the weight of the burette. The stand carrying the apparatus should be securely fixed to the bench. Two pairs of large screw eyelets having two metal meat skewers passed through them will be found effective. Three metal retort rings, each having about an inch of metal removed so as to leave a gap, are arranged to support the levelling bulb at levels 1, 2 and 3, the bulb being connected to the bottom of the apparatus by rubber pressure tubing and the whole filled with mercury. All stop cocks should be air tight and well greased, and preferably of the type with a small spring and washer to prevent the weight of the mercury forcing the taps out, otherwise stout rubber bands should be fitted.

**Collection of Blood** About 10 c c of venous blood are collected

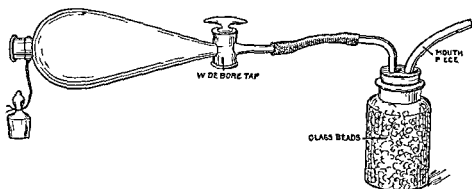


FIG 44 Apparatus for equilibrating plasma with alveolar air

in a syringe containing oxalate and liquid paraffin (see Chapter XVII) without any constriction of the veins. The blood is delivered into a centrifuge tube under 2 or 3 c c of liquid paraffin and centrifuged. About 3 c c of plasma are transferred to a pear-shaped separating funnel, the tap of which should have a wide bore. The funnel is connected to a wash bottle containing moist glass beads (Fig 44), and is held horizontally, as shown.

**Saturation of Plasma with  $\text{CO}_2$**  The air in the separating funnel is displaced by either a 5.5 per cent carbon-dioxide air mixture or by alveolar air from the lungs of the operator. In the latter case, the operator, after a normal inspiration, expires as quickly as possible through the glass beads into the separating funnel, inserting the stopper just before expiration is finished, to prevent atmospheric air from being drawn into the funnel. In order to saturate the plasma the stop cock is closed and the funnel rotated for a few minutes to distribute the plasma as completely as possible over the walls. The funnel is then placed in a vertical position and the plasma allowed to collect above the tap.

**Determination of Carbon Dioxide.** Place the levelling bulb (Fig 43) in position 1, and fill the whole apparatus with mercury, allowing beads of the metal to pass into A and B to act as seals. Close E and lower the levelling tube to position 3. Wait for a few minutes, and then slowly raise the levelling tube. There should be sharp clicks as the mercury in turn strikes up against F and E. Having thus tested the apparatus for leaks, wash out cup B with a few c.c. of 1 per cent ammonia in  $\text{CO}_2$  free distilled water, using a capillary pipette and teat. Remove all but the last trace of the ammonia solution. Remove 1 c.c. of the plasma from the separating funnel with an accurate Ostwald pipette, and deliver into cup B, keeping the tip of the pipette just above the bead of mercury, so that it remains under the surface of the plasma during the delivery. Lower the levelling bulb to position 2, and by careful manipulation of stop cock E, draw in all but the last trace of plasma, but no air. Add a few drops of caprylic alcohol, and very cautiously draw about 1 drop down into the capillary of the stop cock. Then add two  $\frac{1}{2}$  c.c. portions of water, and draw those down in turn into the apparatus, but no air. Lastly, place about 1 c.c. of 20 per cent w/v tartaric acid in cup B, and draw in enough<sup>1</sup> to make the total volume of fluid inside the burette exactly 2.5 c.c. This is checked by closing E and raising the mercury. If the watery fluid does not quite reach the 2.5 c.c. mark, lower the mercury again slightly, open E to B again and suck in the requisite amount of acid. Finally, seal the stop cock by introducing a bead of mercury into the capillary of B with a fine capillary pipette and teat.

Lower the levelling bulb towards position 3, and when the mercury (not the water) has fallen to the 5.0 c.c. mark, close F. Remove the apparatus from its clamps and invert fifteen or more times. Equilibrium of the  $\text{CO}_2$  between the 2.5 c.c. of watery solution and the 47.5 c.c. of free space is thus obtained. Replace the apparatus. With the levelling tube in position 3, turn I to admit all the mercury, and all but the last trace of watery solution, but no gas, into D. Close F. Raise the levelling tube to position 2 and open F to C. Equalise rapidly the levels of mercury in the levelling tube and in the burette, close F, and read off the volume of gas at leisure. *The tiny trace of water should not, of course, be included in the volume of gas read off.*

Note the barometric pressure and the temperature of the room.

**Calculation.** Correct for barometric pressure by multiplying the observed volume of  $\text{CO}_2$  by  $\frac{B}{760}$ , where B equals the barometric pressure.

From the table on p. 201 read off the alkali reserve corresponding to the particular room temperature at which the analysis was made.

This table applies only when 1 c.c. of plasma is used, and when

<sup>1</sup> Half c.c. of 20 per cent w/v tartaric acid is a great excess, there is no risk of incomplete evolution of  $\text{CO}_2$  from  $\text{BHCO}_3$  in the grossest alkalemia. Lactic acid (S.G. 1.2) diluted 1 in 10 with water may be used instead, both are better than dilute  $\text{H}_2\text{SO}_4$ , which was used originally, because they do not coagulate the proteins.

*Calculation of Carbon Dioxide Combining Power of Plasma.<sup>1</sup>*  
(Van Slyke and Cullen)

Observed vol gas B × 760	c c of CO <sub>2</sub> reduced to 0° 760 mm bound as bicarbonate by 100 c c of plasma				Observed vol gas B × 760	c c of CO <sub>2</sub> reduced to 0° 760 mm bound as bicarbonate by 100 c c of plasma			
	15°	20°	25°	30°		15°	20°	25°	30°
0 20	9 1	9 9	10 7	11 8	0 60	47 7	48 1	48 5	48 6
1	10 1	10 9	11 7	12 6	1	48 7	49 0	49 4	49 5
2	11 0	11 8	12 6	13 5	2	49 7	50 0	50 4	50 4
3	12 0	12 8	13 6	14 3	3	50 7	51 0	51 3	51 4
4	13 0	13 7	14 5	15 2	4	51 6	51 9	52 2	52 3
5	13 9	14 7	15 5	16 1	5	52 6	52 8	53 2	53 2
6	14 9	15 7	16 4	17 0	6	53 6	53 8	54 1	54 1
7	15 9	16 6	17 4	18 0	7	54 5	54 8	55 1	55 1
8	16 8	17 6	18 3	18 9	8	55 5	55 7	56 0	56 0
9	17 8	18 5	19 2	19 8	9	56 5	56 7	57 0	56 9
0 30	18 8	19 5	20 2	20 8	0 70	57 4	57 6	57 9	57 9
1	19 7	20 4	21 1	21 7	1	58 4	58 6	58 9	58 8
2	20 7	21 4	22 1	22 6	2	59 4	59 5	59 8	59 7
3	21 7	22 3	23 0	23 5	3	60 3	60 5	60 7	60 6
4	22 6	23 3	24 0	24 5	4	61 3	61 4	61 7	61 6
5	23 6	24 2	24 9	25 4	5	62 3	62 4	62 6	62 5
6	24 6	25 2	25 8	26 3	6	63 2	63 3	63 6	63 4
7	25 5	26 2	26 8	27 3	7	64 2	64 3	64 5	64 3
8	26 5	27 1	27 7	28 2	8	65 2	65 3	65 5	65 3
9	27 5	28 1	28 7	29 1	9	66 1	66 2	66 4	66 2
0 40	28 4	29 0	29 6	30 0	0 80	67 1	67 2	67 3	67 1
1	29 4	30 0	30 5	31 0	1	68 1	68 1	68 3	68 0
2	30 3	30 9	31 5	31 9	2	69 0	69 1	69 2	69 0
3	31 3	31 9	32 4	32 8	3	70 0	70 0	70 2	69 9
4	32 3	32 8	33 4	33 8	4	71 0	71 0	71 1	70 8
5	33 2	33 8	34 3	34 7	5	71 9	72 0	72 1	71 8
6	34 2	34 7	35 3	35 6	6	72 9	72 9	73 0	72 7
7	35 2	35 7	36 2	36 5	7	73 9	73 9	74 0	73 6
8	36 1	36 6	37 2	37 4	8	74 8	74 8	74 9	74 5
9	37 1	37 6	38 1	38 4	9	75 8	75 8	75 8	75 4
0 50	38 1	38 5	39 0	39 3	0 90	76 8	76 7	76 8	76 4
1	39 1	39 5	40 0	40 3	1	77 8	77 7	77 7	77 3
2	40 0	40 4	40 9	41 2	2	78 7	78 8	78 7	78 2
3	41 0	41 4	41 9	42 1	3	79 7	79 6	79 6	79 2
4	42 0	42 4	42 8	43 0	4	80 7	80 5	80 6	80 1
5	42 9	43 3	43 8	43 9	5	81 6	81 5	81 5	81 0
6	43 9	44 3	44 7	44 9	6	82 6	82 5	82 4	82 0
7	44 9	45 3	45 7	45 8	7	83 6	83 4	83 4	82 9
8	45 8	46 2	46 6	46 7	8	84 5	84 4	84 3	83 8
9	46 8	47 1	47 5	47 6	9	85 5	85 3	85 2	84 8
0 60	47 7	48 1	48 5	48 6	1 00	86 5	86 2	86 2	85 7

<sup>1</sup> The temperature figures at the heads of columns represent the room temperature at which the samples of plasma are saturated with alveolar carbon dioxide and analysed. It is assumed that both operations are performed at the same temperature. The figures have been so calculated that, regardless of the room temperature at which saturation and analysis are performed, the table gives the volume (reduced to 0°, 760 mm) of carbon dioxide that 100 c c of plasma are capable of binding when saturated at 20° with carbon dioxide at approximately 41 mm tension. If the figures in the table are multiplied by 0.94 they give within 1 or 2 per cent of the carbon dioxide bound at 37°.



the volume of watery solution in the burette is 2.5 cc. If only  $\frac{1}{2}$  cc of plasma can be employed, use half the volumes of water and acid, making the total volume in the burette 1.25 cc, then multiply the observed gas volume by 2 and use the table. Alternately (but less accurately), if  $\frac{1}{2}$  cc of plasma is diluted to 2.5 cc in the apparatus, convert the observed gas volume into combined  $\text{CO}_2$  with the table and then multiply by 2.

Allowance has been made in the table for the  $\text{CO}_2$  and air in physical solution in the plasma and in the water used in the experiment (see *J. Biol. Chem.*, 1917, 30, 313).

When 1 cc of plasma yields more than 1 cc of gas, repeat with  $\frac{1}{2}$  cc of plasma, making the total volume up to 1.25 cc, multiply by 2, and calculate the alkali reserve,  $x$ , from the formula used in compiling the table, viz.,

$$x = \frac{B}{760} (100.8 - 0.27 t) (V - 0.136 + 0.002 t)$$

where  $t$  is the room temperature, and  $V$  is the observed gas volume.

**Cleaning the Apparatus, and Notes.** Raise the mercury and run out the watery solution as completely as possible through A. Fill B with distilled water, lower the mercury, and wash out the burette, D, C, and both bores of F and E, finally ejecting the water through A by raising the mercury. Repeat with water, then with a few cc of 20 per cent w/v tartaric acid, and then with distilled water again. Then prepare the apparatus and test for leaks as previously described.

After repeated determinations, blobs of mercury and grease, or small lumps of plasma proteins and grease, will adhere to the walls of the vessel. These may be removed by washing, as above described, with 20 per cent w/v tartaric acid, followed by water.

After long use the above method does not suffice. Then 40 per cent sodium hydroxide followed by thorough rinsing with the 20 per cent tartaric acid and water may be tried. Finally, the apparatus will have to be dismantled and thoroughly cleaned. For method of cleaning mercury, see Appendix.

When assembling the apparatus, make certain that the inside of the pressure tubing is really clean. Indiarubber tubing is sometimes stored in chalk ( $\text{CaCO}_3$ ), in which case it must be well washed with dilute 10 per cent v/v hydrochloric acid followed by water. It is more often stored in "French chalk," which is a hydrated magnesium silicate, and should not contain  $\text{CaCO}_3$ .

The pressure tubing should be wired on, or fixed with strong string.

When not in use avoid leaving both taps E and F closed and the apparatus full of mercury, otherwise a rise of temperature by expansion of the mercury may crack the glass.

For interpretation of results see the table on p. 203 and the discussion in the earlier part of this chapter.

(From Hawk and Bergeim's *Practical Physiological Chemistry*)  
*The Relationship of the Plasma Bicarbonate to Acid Excretion, Alkali Tolerance, and Alveolar Carbon Dioxide Tension*<sup>1</sup>

Corresponding Results of Indirect Tests for Acidosis

Condition of Subject	Actual bicarbonate reserve Plasma bicarbonate CO <sub>2</sub> reduced to 0-60 mm.	24 Hour excretion * of $\frac{N}{10}$ acid + $NH_3$		Carbon Dioxide of Alveolar Air		Sodium Bicarbonate required to turn Urine Alkaline	
		(a) c.c. per kg (b) Approx. c.c. per 60 kg person	Reliability in Diabetes	(a) Mm tension (b) Approximate per cent.	Reliability in Diabetes	(a) Cm per kg (b) Approx. gm for a 60 kg person	Reliability in Diabetes
Normal resting adult * Extreme limits of bicarbonate reserve	Vol per cent 77-53	(a) 0-2 (b) 0-1000	Good	(a) 33-35 mm (b) 0.8-4.7 per cent	May indicate some acidosis in its absence	(a) 0-0.5 (b) 0-20	May indicate acidosis in its absence
Mild acidosis no pronounced symptoms	53-40	(a) 7-15 (b) 1000-1000	Good *	(a) 35-27 mm (b) 4.7-3.6 per cent	May indicate more acidosis than is present	(a) 0.5-0.8 (b) 30-50	May indicate much more acidosis than is present
Moderate to severe acidosis Symptoms may be apparent	40-30	(a) 15-100 (b) 4000-6000	liable to considerable error in either direction *	(a) 27-20 mm (b) 3.6-2.7 per cent.	Good	(a) 0.8-1.1 (b) 50-65	
Severe acidosis Symptoms of acid intoxication	Below 30	(a) Over 100 (b) Over 6000		(a) Below 20 mm (b) Below 2.7 per cent	Good	(a) Over 1.1 (b) Over 65	

<sup>1</sup> D. D. Van Slyke, *Jour Biol Chem* 1918, 33, 271

\* Measured either in 24 hour urine or on specimens from shorter period calculated to 24 hour basis

\* After bicarbonate administration likely to indicate more acidosis than is present

\* The figures tabulated in this column also indicate the doses of bicarbonate necessary to restore the alkali reserve to normal from acidosis of the severity indicated by the corresponding plasma CO<sub>2</sub> figures in the first column\* Acidosis (*Amer Jour Dis Child*, 1917, 13, 778) Sells values of 63-46 in the plasma of normal infants & c. about 10 c.c. lower than in adults

### Reaction of Urine to Indicators

Fresh urine is essential. Owing to bacterial decomposition an acid urine may become alkaline in a very short time, *e.g.*, in two to four hours, at room temperature. Bacterial decomposition may be reduced by storage in an ice room, but such treatment is not a certain preventive of changes in *pH*. Toluene and other preservatives will lessen bacterial activity, but no preservative is certain in its action (see Chapter XVI). Much depends on whether the original urine is already infected with organisms, and what type of organism is present.

When watching the influence of alkaline treatment the early morning specimen should always be tested, because the night's urine is the most difficult to keep alkaline. Litmus paper is satisfactory. So long as it turns blue (*pH* greater than 7.0) the dose of alkali is certainly large enough, but it may be too large. It is a good plan, therefore, to use also phenol red. If this turns purple red (*pH* about 7.8 or higher) it is possible that too much alkali is being given, and the dose should be reduced.

Apart from controlling treatment by alkali or by acid, testing the reaction of the urine is not of much use clinically, because the normal range of *pH* is so large, *viz.*, 4.8 to 7.4 (Wilson). As a rough test of the acidity brom cresol purple (range of *pH* 5.2 to 6.8) and methyl red (range of *pH* 4.4 to 6.0) may be used (Tallerman).

To about 5 c.c. (1 in. column) of urine in a test tube add 5 drops of brom cresol purple (about 0.2 per cent aqueous solution). If the colour is purplish no further tests need be carried out. The *pH* is on the alkaline side of 6.2. If a yellow colour is obtained to another 5 c.c. of urine add 5 drops of methyl red (about 0.2 per cent in 60 per cent alcohol). If the colour then obtained is yellow orange and not a definite pink the urinary acidity lies to the alkaline side of *pH* 5.0 and the urine cannot be abnormally acid.

In health, and in most pathological states in which the kidneys are sound, if the urine is not abnormally acid as judged by the above tests, there is either no acidosis or no danger from an acidosis. But in certain pathological states and particularly when the kidneys are diseased it is unsafe to rely on the above tests.

If the urine turns pink with methyl red, it is unduly acid, and the existence of an acidosis is probable.

Recently the determination of the actual *pH* of the urine has become of great clinical importance in controlling the treatment of urinary infections by ketogenic diet or by mandelic acid and "acid salts" (*e.g.*, ammonium chloride)—see Chapter XVI—so that in many laboratories the above rough tests have been replaced by colorimetric measurement of *pH* (see p. 295).

### Bicarbonate Tolerance Test (Sellard's)

Sodium bicarbonate is administered until the reaction of the urine becomes alkaline. The number of grammes required per kgm of body weight is noted.

Instruct the patient to empty the bladder completely. Give by

mouth 5 gm of sodium bicarbonate in 100 c c of water. Collect the urine at the end of half an hour and repeat the dose of bicarbonate. Continue this process, testing each sample of urine on the spot, until the urine becomes alkaline to litmus.

Calculate the number of grammes of bicarbonate required per kgm of body weight.

When the urine becomes only faintly acid, boil the sample so that  $\text{CO}_2$  is driven off and bicarbonate becomes converted into carbonate. If the boiled urine is alkaline to litmus, stop the bicarbonate.

Avoid giving bicarbonate once the urine has become alkaline, so as to run no risk of serious alkalosis.

The method is trustworthy for showing the absence of acidosis, but if acidosis is present the test may indicate a more serious grade of acidosis than actually exists. Normally 0 to 0.5 gm of bicarbonate per kgm will turn the urine alkaline. Usually a total of 5 to 10 gm suffices, but for fuller data see the table on p. 203.

### References to and Notes on other Technical Methods

**General.** An extensive account of technique is given in Peters and Van Slyke's *Quantitative Clinical Chemistry*, Vol. II, and in Hawk and Bergeim's *Practical Physiological Chemistry*, chapter on Respiratory Metabolism and Neutrality Regulation.

**Alveolar  $\text{CO}_2$ .** Haldane Priestley Method. Haldane, J. S., and Priestley, J. G., *J. Physiol.*, 1905, 32, 225. *Recent Advances in Medicine*, Beaumont G. E., and Dodds E. C., chapter on Glycosuria and Diabetes Mellitus. This is the most accurate method, but necessitates skill in gas analysis.

**Electrometric (Katharometer) Method.** Rabinowitch, I. M., and Bazin E. V., *Canad. Med. Ass. J.*, 1926, 16, 638.

**Blood pH.** This may be determined colorimetrically, electrometrically or indirectly from  $\text{CO}_2$  tensions and volumes, using Hasselbalch's formula. For references the reader is referred to Austin and Cullen's book. The following are additional references:—

Cole, S. W., *Practical Physiological Chemistry*, Chapter I (Direct reading potentiometer).

Kerridge, P. T., *Biochem. J.*, 1925, 19, 611, and *J. Sci. Inst.*, 1926, 3, 464 (Glass electrode).

Martin, C. J., and Lepper, E. H., *Biochem. J.*, 1926, 20, 37 (Micro method capillary blood).

Meeker, G. H., and Oser, B. L., *J. Biol. Chem.*, 1926, 67, 307 (See also Hawk and Bergeim's book).

Pope, C. G., *Brit. J. Exper. Path.*, 1928, 9, 225 (Simple electrometric comparator).

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**Alkali Reserve (Manometric Method).** Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, 61, 523, and Peters and Van Slyke's second volume.

**Plasma Bicarbonate (Titration Method).** Van Slyke, D. D., *J. Biol. Chem.*, 1922, 52, 495.

Lepper, E. H. and Martin, C. J., *Biochem. J.*, 1925, 19, 573.

**Total Bases of Blood.** Van Slyke, D. D., Wu, H., and Maclean, F. C.,

*J. Biol Chem*, 1923, 56, 765 Stadie, W. C, and Ross, E C, *J Biol Chem*, 1925, 65, 735 Van Slyke, D D, Hiller, A, and Berthelsen, K. C, *J. Biol Chem*, 1927, 74, 659

**Ammonia Coefficient of Urine.** Strictly speaking, determinations of (a) the total nitrogen (Kjeldahl method) and (b) the ammonia nitrogen (see books on Chemical Physiology) should be made The coefficient equals

$$\frac{\text{Ammonia N percentage}}{\text{Total N percentage}} \times 100$$

In clinical work an estimation of urea N is often substituted for total N because the technique for urea is so much simpler The coefficient then becomes

$$\frac{\text{Ammonia N percentage}}{(\text{Urea N} + \text{Ammonia N}) \text{ percentage}} \times 100$$

Since urea N generally forms 80 to 90 per cent of the total N, this method seldom introduces serious error If, however, high coefficients are obtained, the more accurate method should be used Normally the coefficient is 3 to 5 per cent It is essential to preserve the urine efficiently (see Chapter XVI) If ammoniacal decomposition of the twenty hours' urine by organisms is not prevented, absurdly high coefficients may easily be obtained

**Total Acid plus Ammonia in the 24-Hours' Urine (Index of Acid Excretion)** Fitz, R, and Van Slyke, D D, *J Biol Chem*, 1917, 30, 389 Van Slyke, D D, *J Biol Chem*, 1918, 33, 271 For interpretation of results see the table on p 203

**Total Organic Acids in Urine.** Van Slyke D D, and Palmer, W W, *J Biol Chem*, 1920, 41, 567 McCluskey, K L, *J Biol Chem*, 1931, 90, 197

**Estimation of Acetone Bodies in Blood and in Urine.** Van Slyke, D D, *J Biol Chem*, 1917, 32, 455 and 495, and 1929, 83, 415, and in books on Physiological Chemistry, e g, Cole's

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 TALLERMAN, K H *Arch Dis Child*, 1926, 1, 50  
 VAN SLYKE, D D *Physiol Rev*, 1921, 1, 141 (See also Chapter XIX of Hawk and Bergheim's book)  
 WILSON, D W *Physiol Rev*, 1923, 3, 318  
 WOODYATT, R T *Arch Int Med*, 1921, 28, 125

## CHAPTER X

### BLOOD AND ITS DERIVATIVES IN THE URINE

**Books and References** The books on Physiological Chemistry, Clinical Pathology and so on mentioned at the beginning of previous chapters should be consulted but the writer has failed to find any one comprehensive account of methæmoglobinuria. Methæmoglobin and sulphæmoglobin have recently been more fully studied in the blood, so to avoid repetition the references are given at the end of Chapter XVIII. Congenital porphyria is exhaustively reviewed in Garrod's *Inborn Errors of Metabolism* to which the reader is referred for references. The writer's account is an extract of Garrod's excellent chapter. Mackey and Garrod have published further observations (*Quart J Med*, 1926, 19, 357, 1936 29, 473).

For a general discussion of the use of the spectroscope, and in particular its application to the study of blood and other pigments, see MacMunn's *The Spectroscope in Medicine* and *Spectrum Analysis applied to Biology and Medicine*.

The relative value of clinical tests for blood in urine has been studied by Bloem (*Biochem J*, 1933, 27, 121).

THE discovery of proteinuria (Chapter III) led automatically to the study of urinary deposits (Chapter IV), and was extended by the application of renal efficiency tests (Chapter V). Similarly, reducing substances in the urine (Chapter VI) were followed by blood sugar tests (Chapters VII and VIII), and a consideration of ketosis, acidosis and alkalosis (Chapter IX). The next subject for discussion is the presence of blood and its derivatives in the urine.

As mentioned in Chapter II, blood will either be suspected from the colour of the urine, or hæmaturia will be demonstrated by the finding of red blood corpuscles in the centrifuged deposit. Routine tests for blood have been given in Chapter II, and means of identifying erythrocytes in Chapter IV.

Stress has already been laid on the fact that proteinuria must necessarily accompany hæmaturia or hæmoglobinuria, and that an examination of the centrifuged deposit for red cells is a more delicate test for hæmaturia than any chemical test. Indeed, the writer has for several years discarded all chemical tests in favour of microscopical examination of the urinary deposit (hæmaturia) or spectroscopical examination (hæmoglobinuria). For other causes of red and allied colours see Chapter XI. Methæmoglobin is a protein (*cf* Chapter XVIII) and so proteinuria must accompany methæmoglobinuria, but the porphyrins are not proteins (*cf* Chapter XVIII), and proteinuria does not, as a rule, accompany porphyrinuria.

In clinical work blood and its derivatives in the urine include

hæmaturia, hæmoglobinuria, methæmoglobinuria and porphyrinuria. Bilirubin is also a hæmoglobin derivative, but is considered more conveniently under bile in urine (Chapter XII)

The direct vision spectroscope plays an important part in the detection of hæmoglobin and its derivatives, and will now be described

### *The Direct-Vision Spectroscope*

This is a most useful little instrument for clinical work. It assists in the identification of a number of substances, particularly hæmoglobin and its derivatives. The larger angular vision spectrometer, though of more value in special investigations, is too elaborate and requires too much preparation for routine work.

The instrument (Fig 45) is fitted with an adjustable eye piece, E, at one end and a movable slit, S, at the other<sup>1</sup>. It is advisable to employ daylight, so as to be able to "place" the absorption bands in relation to the Fraunhofer lines, as well as in relation to the different colours of the spectrum. With the best types of direct



FIG 45 Direct vision spectroscope. E. Eye piece S Milled edge for adjusting slit C Cover

vision spectroscope, supplied with a wave length scale, it is possible to obtain an approximate measurement of the wave lengths of the absorption bands, but for accurate measurements the larger spectrometer or a calibrated reversion spectroscope is required. In routine work it is seldom necessary to make such measurements.

### *Instructions for Use*

(a) Place the eye piece E to the eye, and view a bright part of the sky, but avoid direct sunlight.

(b) Close the slit S and then open it slightly, so that the colours of the sun's spectrum are just visible.

(c) Pull out or push in the eye piece E carefully until the colours become clear, and until a series of vertical lines (Fraunhofer lines, see below) becomes sharply defined. The instrument is then in proper focus.

Sometimes horizontal lines are visible. These are due to specks of dust on the edges of the slit. If they are annoying open the slit just a tiny bit more. If they do not disappear the slit requires cleaning with a fine camel hair brush or a piece of hard wood cut into a smooth thin wedge.

<sup>1</sup> Several firms supply a pocket spectroscope for students, costing 21s. to 23s. This type of spectroscope is of the grating pattern so that the dispersion of the colours is a little different from that indicated in Figs 47 and 48 and it has a fixed slit. It is satisfactory for most purposes.

Do not force the jaws of the slit apart roughly. The milled edge requires just a slight gentle twist through a very few degrees. The instrument is fairly delicate and will not stand rough handling.

(d) Place the solution to be examined in a test tube, or, better, in a glass vessel with parallel sides<sup>1</sup>. For routine purposes an ordinary  $\frac{5}{8}$  in test tube, or a 1 in boiling tube, is quite satisfactory, but often a conical urine receiver is the best of all because layers of different thickness may be examined.



FIG 46 The direct vision spectroscope in use. The test tube is kept in position by resting it and the end of the instrument against the tip of the fourth digit.

With an ordinary test tube a thick layer may be viewed by placing the spectroscope vertically above the tube, and looking down through the length of the tube which is held above an electric bulb. This may usefully be practised as a routine but is especially valuable when the volume of solution is limited to some 5 to 10 c.c. (see Fig 46A).

(e) Place the tube in front of the slit. A convenient method of resting it against the finger (fourth digit) is illustrated in Fig 46. Look through the spectroscope and record the position of the absorption bands (if any) in reference both to the colours of the spectrum and to the Fraunhofer lines.

<sup>1</sup> Small glass cells of this type and of varying thicknesses are made by Messrs The Tintometer Ltd. Milford Salisbury.



Charts showing absorption bands are illustrated in Figs 47 and 48. These charts have been specially drawn (approximately to

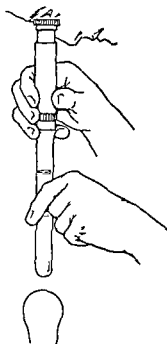


FIG. 46A. Tube viewed from above to obtain a thicker layer

scale) to illustrate what is visible in the clinical examination of blood, urine or faeces using a direct vision prismatic spectroscope and represent the relative position of the bands as seen. The vertical lines (Fraunhofer lines) in the sun's spectrum are due to absorption of light by different elements in the sun's atmosphere, and are given below

*Wave lengths of Fraunhofer Lines (in tenth metres)*

Line	Part of Spectrum	Element to which Line corresponds	Wave length $\lambda$ 0.1 $\mu$
B	Red	Oxygen	6 867
C	Red	Hydrogen	6 563
D	Orange	Sodium	5 896 and 5 890
E	Green	Iron and calcium	5 270
b	Green	Magnesium	5 184
F	Blue	Hydrogen	4 861
G	Violet	Iron and calcium	4 308

B and G are easily seen, or are seen with difficulty or are not visible according to the efficiency of the spectroscope. G, F and b are the thickest lines, D is nearly as thick, and E, C and B are about equally thin.

Wave lengths ( $\lambda$ ) are usually expressed in "tenth metres" ( $10^{-10}$  m) or in Ångström units. An Ångström unit (Å) is very nearly equivalent to 1 tenth metre, and is generally regarded in practice as the same. Sometimes the wave lengths are given in terms of  $10^{-9}$  m, =  $10^{-6}$  mm, =  $m\mu$ . Thus the  $\alpha$  band (nearer the D line) of oxyhæmoglobin has its centre at  $\lambda = 5,780$  tenth-metres (or Å), or  $\lambda = 578m\mu$  (see table on p. 315). The absorption bands are lettered  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  from the left or red end progressively to the right or violet end.

If direct examination of the urine shows no absorption bands, examination of an amyl alcohol extract after acidification of the urine with acetic acid may be more successful, but care is then necessary in interpreting the results since some pigments may be so concentrated thereby that even normal amounts may yield absorption bands (see under descriptions of individual pigments). It should be mentioned also that the use of different solvents may shift slightly the position of the bands.

### Hæmaturia

The causes of hæmaturia are numerous and are fully described in the text books of medicine and surgery. Clinically it is important to decide whether the hæmaturia is accidental due to contamination, and particularly by the menses or true, due to red cells passing into the urine from some part of the urinary tract.

The hæmaturia may be microscopic not enough red cells in suspension to make the colour abnormal, or macroscopic when the mixed urine is red, pale red or smoky. Obviously the intensity of colour depends on the thickness of the layer examined, urine definitely red in bulk, as in a Winchester quart bottle, may be normal in colour when viewed in a test tube. Again, a  $\frac{5}{8}$  in thickness of urine in a tube may be normally coloured, but when 10 c.c. are centrifuged red cells may be obvious to the unaided eye as a red deposit. This may be illustrated by diluting 1 c.c. of normal blood, to between 5,000 and 50,000 c.c. with normal urine.

Lastly, centrifuging may yield a deposit which is not red but which contains red cells in amounts which are easily seen under the microscope. This may be illustrated by diluting 1 c.c. of normal blood to between 100,000 and 600,000 c.c. with normal urine. Bloem has confirmed the statement that the most satisfactory test for hæmaturia is the microscopical examination of the centrifuged deposit.

It is not a common practice to use the spectroscope to detect hæmaturia but if the red cells are suspended evenly by mixing, this instrument will show both the  $\alpha$  and  $\beta$  bands of oxyhæmoglobin in a layer 1 in. thick when 1 c.c. of normal blood is diluted to about 1,250 c.c. with normal urine. If a sample of mixed urine (1 in.

thick or less) is red, and the bands of oxyhæmoglobin are not visible, the red colour cannot be due to oxyhæmoglobin.

The chemical catalytic tests are more sensitive than the spectroscopic test as above defined, but are not nearly so sensitive as the microscopical examination of the centrifuged deposit for red cells, and are not specific. As commonly performed in clinical work, the reduced phenolphthalein and pyramidone tests (*cf.* Chapter II) are just positive when 1 c.c. of normal blood is diluted to about 4,000 c.c. with normal urine.

#### NOTES ON FIG. 47

The wavelengths of the Fraunhofer lines, B to G, are given in the table on p. 210. The wavelengths of the absorption bands in Fig. 47 are recorded on p. 315.

*Oxyhæmoglobin and Carboxyhæmoglobin.* When in more concentrated solutions the  $\alpha$  and  $\beta$  bands are fused, and when still more concentrated only the red part of the spectrum is visible.

*Methæmoglobin.* In a slightly more concentrated solution the  $\gamma$  and  $\delta$  bands cannot be distinguished, and are fused with the general absorption of the violet. In laked blood, owing to the presence of oxyhæmoglobin, only the  $\alpha$  band of methæmoglobin is visible in a manner similar to that illustrated for sulphæmoglobin.

*Alkaline Methæmoglobin.* In a more dilute solution only the  $\beta$  and  $\gamma$  bands in the green are seen.

*Sulphæmoglobin* alone in water shows, in addition to the  $\alpha$  band, two bands  $\beta$  and  $\gamma$  in the green (*cf.* tables below and on p. 315).

*Porphyrins (alk. and acid)* These are group spectra. There are small differences in the positions of the bands of the different porphyrins (*cf.* p. 315).

It is of material assistance in the recognition of the above spectra, and when converting one derivative into another, to know the relative intensities of the absorption bands. Thus, that oxyhæmoglobin is present in addition to methæmoglobin can be deduced from the observation that the  $\beta$  and  $\gamma$  bands are more intense, relative to the  $\alpha$  band, than would be the case for methæmoglobin alone. Again, a dilute solution which initially shows the two bands of oxyhæmoglobin after reduction may give no band at all, because the intensity of the band of reduced hæmoglobin is much less than that of the oxyhæmoglobin bands.

Bloem's results, in which the intensity of the  $\alpha$  band of oxyhæmoglobin is taken as the standard and is called 100, are as follows—

*Relative intensities (approximate) of absorption bands of hæmoglobin derivatives ( $\alpha$  band of oxyhæmoglobin = 100).*

Absorption band	$\alpha$	$\beta$	$\gamma$	$\delta$
Oxyhæmoglobin	100	57	—	—
Carboxyhæmoglobin	63	45	—	—
Sulphæmoglobin	10	35	16	—
Reduced hæmoglobin	10	—	—	—
Methæmoglobin	9	5	7	?
Acid hæmatin	7	2	6	?
Alkaline hæmatin	3	—	—	—
Hæmochromogen	173	25	—	—
Acid porphyrin	14	47	—	—
Alkaline porphyrin	7	13	20	?

#### Hæmoglobinuria

In the great majority of instances hæmoglobin gains access to the urine enclosed in red corpuscles (hæmaturia), *i.e.*, there is hæmorrhage of greater or less extent into some part of the urinary passages. In a few conditions, notably paroxysmal hæmoglobinuria,

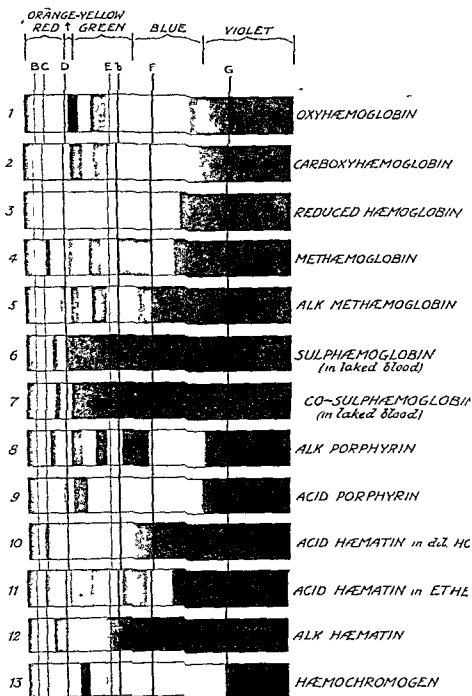


FIG 47. Absorption spectra of hæmoglobin and its derivatives.

blackwater fever, and after the transfusion of incompatible blood, hæmoglobin is liberated into the plasma (hæmoglobinæmia, see Chapter XVIII), and is then excreted by the kidneys in solution (hæmoglobinuria)

"False hæmoglobinuria" may result from urine containing red cells being allowed to stand for some time. Some of the erythrocytes become ruptured, with consequent liberation of their contents. This may be due to hæmolysins or to alteration of the reaction of the urine owing to bacterial decomposition. At any rate, fresh urine is usually not sufficiently hypertonic or hypotonic to cause rupture of red cells, though it may cause shrinkage and crenation, or swelling with loss of the double contour (*cf* Chapter IV)

Depending on the amount of hæmoglobin, the urine may be obviously red, or of a "smoky" tint, or apparently normal in colour. Hæmoglobin is readily detected by the characteristic spectrum of oxyhæmoglobin (Fig 47). If the urine is red, and this spectrum is not visible on examination with a spectroscope, it is certain that the red colour is not due to hæmoglobin. If no absorption bands are seen when a layer, 3 in. or 4 in. thick, of urine which has been cleared by centrifuging is examined, no significant degree of hæmoglobinuria is present.

### Methæmoglobinuria

Methæmoglobinuria may be "true" or "false." Almost any acid urine containing oxyhæmoglobin will, after standing for some time, show the absorption bands of methæmoglobin. True methæmoglobinuria may occur in any of the conditions in which methæmoglobin is found in the plasma. It has been reported in severe toxic or septicæmic conditions where it may be marked, in phenylhydrazine poisoning, in blackwater fever, after the transfusion of incompatible blood, and in some cases of paroxysmal hæmoglobinuria (*cf* Chapter XVIII)

The urine is brown, pale brown, or of normal colour, depending on the concentration of the pigment. The diagnosis depends on observation of the absorption spectrum (Fig 47) and the action of reducing agents, or of alkali on that spectrum. The absorption bands are not intense (*cf* p 212), and unless a thick layer is viewed it may be difficult to see the characteristic bands even though the urine is brownish. It is recommended that a 4 in. layer be examined before declaring that methæmoglobin is absent. Using a 1 in. thickness, Bloem could just detect all four bands when the urine contained about 0.11 gm. per 100 c.c. The  $\alpha$  band in the red is the most characteristic, and is removed by the following chemical treatment. With reducing agents (yellow ammonium sulphide, Stokes' reagent or solid sodium hydrosulphite,  $\text{Na}_2\text{S}_2\text{O}_4$ ) the spectrum of methæmoglobin is replaced first by that of oxyhæmoglobin and then by that of reduced hæmoglobin.

If the original urine is made alkaline with concentrated ammonia, alkaline methæmoglobin is formed which has two bands in the green

and no band in the red<sup>1</sup>, the positions of the two bands ( $\beta$ ,  $\gamma$ ) closely resemble those of oxyhæmoglobin, but the  $\gamma$  band is the more intense (Fig 47), it is important to remember this if the original urine is already alkaline

### Sulphæmoglobinuria

Theoretically sulphæmoglobin might be excreted in the urine in those cases (anærobic sepsis) in which sulphæmoglobin occurs in the plasma (see Chapter XVIII), but the writer knows of no record of such a case

In a case of gross methæmoglobinuria due to septicæmia (*B. Welchii*), the writer noted that on adding ammonium sulphide to the urine, the band in the red disappeared in the usual way, but that another weak band appeared in the red in the position of the sulphæmoglobin band. Professor Snapper in a personal communication confirms this, and informs me that it is not very rare in cases of methæmoglobinuria to convert some of the methæmoglobin into sulphæmoglobin by this treatment

### Porphyrinuria

There are two porphyrins which may occur in urine, uroporphyrin and copro- or sterco porphyrin. The spectra of the two are very similar and can only be differentiated by a refined technique. Protoporphyrin, prepared from blood, likewise is very similar spectroscopically (for the wavelengths of the bands of the three porphyrins see table on p 315), and the three cannot be differentiated by the simple direct vision spectroscope, so that a "group spectrum," alkaline or acid, is illustrated in Fig 47

Porphyrin (coproporphyrin) occurs normally in traces insufficient to affect the colour of the urine, or to yield an absorption spectrum. A slight increase over the normal trace, with no effect on the urinary colour, occurs in many diseases, but will not be considered here (cf p 220)

When present in gross quantities porphyrins make the urine dark port wine in colour

A mixture of methæmoglobin and oxyhæmoglobin also may impart a port wine colour, but is easily differentiated by the spectroscopical changes on adding a reducing agent ( $\text{Methb} \rightarrow \text{HbO}_2 \rightarrow \text{Hb}$ , porphyrin unaffected) or a mineral acid ( $\text{Methb}$  and  $\text{HbO}_2 \rightarrow \text{acid hæmatin}$ , alkaline  $\rightarrow$  acid porphyrin)

The porphyrins may be precipitated from urine by treating each 100 c.c. with 5 c.c. of glacial acetic acid, and allowing the mixture to stand. They are excreted as alkaline porphyrins (Fig 47), and continue to give the spectrum of the alkaline pigments when treated with acetic acid, but yield acid porphyrins with hydrochloric acid (Fig 47). Sometimes the original urine shows an atypical two banded spectrum, very similar to that of oxyhæmoglobin

<sup>1</sup> In relatively concentrated solution alkaline methæmoglobin shows also a poorly defined band ( $\alpha$ ) at the junction of the red and orange (Fig 47) for the wavelengths of these bands see p 315

This may be due to the porphyrin being in the form of a metal complex (two banded or metallic<sup>1</sup> spectrum of Stokvis, Hammarsten, and Garrod), but it should be remembered that a somewhat similar spectroscopic appearance is seen on examining a solution so dilute that the least intense (*cf* p 212)  $\alpha$  band is not, but the other bands are visible (Bloem). As a routine, therefore, before concluding that porphyrin is absent, the urine should be acidified with acetic acid and extracted with amyl alcohol, after which the extract is acidified with HCl and examined spectroscopically as described in detail below.

Porphyrinuria occurs in pathological amounts in three main classes of patient (a) those susceptible to certain drugs, viz., sulphonal, trional, veronal, etc., (b) those with the inborn error of metabolism, "congenital porphyrinuria", (c) those with acute idiopathic porphyrinuria not due to drugs.

*Sulphonal and its allies have been used extensively as hypnotics, particularly in asylum practice, but porphyrinuria from their use is uncommon. Moreover, some patients tolerate large doses over long periods, whereas others develop the urinary condition after doses well within the therapeutic range. There would appear, therefore, to be a peculiar sensitivity to the drug in these cases. The porphyrinuria as a rule only develops after treatment has lasted several weeks or months. The porphyrin in the urine is uroporphyrin  $C_{40}N_3S_4O_{16}$ .*

Congenital porphyrinuria or porphyria, probably present at birth, is very rare. The porphyrin sensitises the patient to light, with the result that hydroa aestivale occurs on the exposed parts, and this may lead in time to extensive scarring. The parts covered with hair escape, and in some patients marked hirsutes develops. Not all cases of hydroa aestivale, however, have been shown to have porphyrinuria, and not all cases of porphyrinuria develop hydroa. Loss of nails, deformities of the hands, nose and ears, and blindness may follow. The pigment is deposited in the bones and in the teeth in some cases, and transillumination of the hands may prove a useful method in diagnosis. Fischer found that the chief porphyrin in the urine (uroporphyrin  $C_{40}H_{38}N_4O_{16}$  about 0.3 gm daily) differed from that in the faeces (stercoporphyrin  $C_{36}H_{38}N_4O_8$  about 0.1 gm daily). *The urine might contain a little stercoporphyrin as well as uroporphyrin, but the faeces contained only stercoporphyrin.* The porphyrins nowadays are regarded as intermediate products in the building up of hæmoglobin, and the lack of an enzyme responsible for one of the stages of this synthesis may be the cause of congenital porphyria. Alternatively, as Fischer suggests, the porphyrins may be regarded as vestigial, coproporphyrin being very widespread in the animal and vegetable kingdoms. In Fischer's view they are not derived from hæmoglobin. Fischer found that uroporphyrin was more toxic, but that stercoporphyrin induced greater sensitiveness to light. On this

<sup>1</sup> A similar spectrum is obtained by treating a solution of a porphyrin with zinc chloride and ammonia.

hypothesis it becomes important to examine the faeces for excess of porphyrin in hydroa. The pigment may exist in urine and faeces as a colourless chromogen, porphyrinogen, which can be converted into the porphyrin in a few minutes by heating at  $100^{\circ}\text{C}$ , or slowly by the action of light. This may be one explanation of the intermittent appearance of the porphyrins in the excreta, but it requires further investigation.

When urine containing porphyrin is examined with filtered ultra violet rays it shows a beautiful pink fluorescence. Mackey and Garrod found that the teeth of their patient (with pink teeth) shone out with a brilliant pink light when brought into the path of the ultra violet rays, and they record other interesting observations with the fluorescence test on the sections of the teeth.

### Simple Clinical Tests for Porphyrinuria.

(a) Examine the urine spectroscopically for alkaline porphyrin. Make the urine strongly acid to litmus by adding a few drops of concentrated  $\text{HCl}$ , and examine for the absorption bands of acid porphyrin (Fig. 47). This test will often suffice in urines of a port wine colour, but will generally not detect porphyrin in urines not abnormal in colour unless a very thick layer of urine be examined. Gunther suggests that if a layer 2 in. thick shows the bands of acid porphyrin, a pathological amount of the pigment is present.

(b) In a centrifuge tube place about 15 c.c. of urine, a few drops of glacial acetic acid so as to make the reaction strongly acid to litmus, and about 5 c.c. of amyl alcohol. Mix well and centrifuge. Examine the amyl alcohol layer for the absorption bands of alkaline porphyrin. Separate the amylic extract and to it add 1 or 2 drops of concentrated  $\text{HCl}$ . Shake and examine for the bands of acid porphyrin, which are about twice as intense as those of alkaline porphyrin (cf. p. 212).

The amylic extract must be separated before adding  $\text{HCl}$ , otherwise the acid porphyrin will pass into the aqueous layer.



## CHAPTER XI

### URINES ABNORMAL IN COLOUR. DRUGS IN URINE

**Books, etc.** Descriptions of the substances responsible for abnormal colours of the urine are scattered through the textbooks of chemical physiology, clinical pathology, chemical pathology and medicine, but in no single book is there a really comprehensive account. The maximum information may be obtained from Garrod's publications (e.g., *Edin Med J*, 1897, 2, 105, *Lancet*, 1900, 11, 1323). In Dixon Mann's *Physiology and Pathology of the Urine* (London, 1904) there is a good chapter on uncommon adventitious pigments and drugs in the urine, part of which the writer has abstracted.

For fuller accounts of drugs and poisons in urine the reader is referred to the standard works on Pharmacology, and Forensic Medicine and Toxicology, together with the more clinical books referred to above. Wynter Blyth's *Poisons Their Effects and Detection*, and Autenrieth's *Laboratory Manual for the Detection of Poisons and Powerful Drugs* (translated by W. H. Warren), may especially be recommended. In the following account, the discussion has been confined to simple tests and the points of clinical importance in dealing with material obtained during life.

THE various substances and conditions which may cause the urine to depart from the normal amber colour are listed below. These may be grouped under the following headings —

- (a) Physiological variations in colour
- (b) Blood and its derivatives, which have already been discussed in Chapter X
- (c) Bile pigments and urobilin. These will be reviewed in Chapter XII
- (d) Miscellaneous pathological conditions
- (e) Drugs, which will be considered in the second part of this chapter

#### Colours of Urine

Colour	Causes
Amber	Normal
Nearly colourless	Large fluid intake    Reduction of perspiration Chronic nephritis (interstitial) Untreated diabetes mellitus Diabetes insipidus Alcohol    diuretics and nervousness Concentrated urine (restricted fluid intake, sweating)
Orange.	Fevers Urobilin Small quantities of bile pigments Pyridium and allied drugs
Orange reddish brown	Certain drugs (rhubarb, senna)
Dark brown	Altered blood (methæmoglobinuria, false and true) Phenolic drugs (phenol, cresol, phenylhydrazine)

<i>Colour</i>	<i>Causes</i>
<i>Red</i>	Blood Pyramidone (amidopyrine) Pyridium Neotropin Prontosil. Aniline dyes (sweets) Beetroot (anthocyaninuria)
<i>Purple red</i>	Phenol red and phenolphthalein (purgen) in alkaline urine.
<i>Port wine</i>	Porphyrin <i>e.g.</i> , sulphonal congenital porphyria Mixture of methæmoglobin and oxyhæmoglobin
<i>Brownish black</i>	Much hæmoglobin Marked carboluria and Ivaol poisoning <i>Melana</i> Alkaptonuria
<i>Greenish</i>	Bile pigments Methylene blue Indigo carmine Carbolic acid guaiacol santonin
<i>Blue</i>	Flavine (bladder wash out) Methylene blue Indigo blue.

### THE PIGMENTS OF NORMAL URINE

Urochrome is the chief pigment, and is responsible for most if not all the yellow colour of normal urine. It yields no absorption band. In addition, normal urine contains traces of urobilinogen, coproporphyrin, and possibly uroerythrin. Urobilinogen is the colourless chromogen of urobilin. On exposure to light and air it changes into urobilin, and in concentrated urines (*e.g.*, after sweating) it is possible that this change may, in part at any rate, be responsible for the slight intensification of the colour of the urine on standing. Coproporphyrin and uroerythrin occur in such small traces that they probably do not contribute at all to the colour of normal urine (Garrod, 1900). There may be a minute trace of bilirubin (see p. 235).

### PHYSIOLOGICAL VARIATIONS IN COLOUR

The urine may be pale or almost colourless, owing to the intake of large volumes of fluid or to the reduction of perspiration on a cold day.

It may be deeper in colour, more orange than the average normal, owing to copious sweating or to reduction of fluid intake. Such a urine is frequently referred to as "concentrated."

The urine of healthy individuals may exhibit unusual yellow, orange, or reddish tints, owing to the ingestion of sweets coloured with aniline dyes, or other pigments. Urines containing such dyes or their products may exhibit colour changes on the addition of acid or alkali. In other words, the colouring matter of sweets, or their excretory products, may act as indicators. In clinical work when such colour changes are noted, the simplest procedure is to make inquiries as to the ingestion of sweets, etc., and then to observe the effect of stopping the ingestion of the suspected article on the colour of the urine. Sometimes a solution of the sweet in water may give the same colour changes, on addition of acid or alkali, as the urine itself. But in other cases the dye is altered in its passage through the body, and the colour changes in the urine are

due to this altered product, and not to the dye originally ingested. Obviously in that case chemical tests on a solution of the sweet may be misleading or of no value. The observer may be able to reproduce the unusual colour in his own urine by ingestion of the suspected article, and to make experiments accordingly, but not uncommonly, in the writer's experience, this fails, owing either to the difficulties of reproducing the conditions existing for the "patient," or to the presence of some unknown peculiarity in the "patient's" intestinal absorption, or metabolism, and so on.

Eosin is an example. It imparts to the urine a pinkish red tint with a green fluorescence. It may be extracted with amyl alcohol. The extract gives an absorption band in the green extending from half way between D and E up to E. In the original urine, if there is a sufficient concentration of eosin, the band is nearer the violet and lies between E and b, slightly overlapping both lines (Fig. 48). On adding a few drops of 10 per cent v/v HCl to the amyl extract or urine, the colour and the absorption band disappear, to reappear on making alkaline with NaOH.

Very occasionally the ingestion of common articles of diet (beetroot, bilberries, blackberries, etc.) lead to peculiar colours of the urine. Anthocyaninuria, due to the ingestion of red beet (*cf.* Poole) is an example. The writer observed a case in a boy of seven (with diabetes mellitus) due to beetroot. The fresh urine was acid and was orange pink. It turned canary yellow when made alkaline with sodium hydroxide, to become pink again when made acid with HCl. Spectroscopically it showed no absorption bands, and contained no red cells and no hæmoglobin. On discontinuing the beetroot the pink colour stopped, to reappear after giving beetroot again. A dilute watery extract of beetroot gave similar colour changes with alkali and acid. The pigment was not extracted from the urine by amyl alcohol.

### COLOUR CHANGES DUE TO MISCELLANEOUS PATHOLOGICAL CONDITIONS

The urine is pale or almost colourless in untreated diabetes mellitus, chronic interstitial nephritis, and diabetes insipidus. In untreated diabetes mellitus the specific gravity is high (*e.g.*, 1,025 to 1,040) whereas in the other two diseases it is low (*e.g.*, 1,002 to 1,010). The same dilution of pigment may occur in any of the causes of polyuria, such as that due to alcohol, diuretics and nervous excitement.

The urine is often more deeply pigmented in disease than normally, simply owing to concentration. This may be due to a variety of causes, restricted intake of fluid due to anorexia, increased sweating as in fevers, poor circulation as in heart disease, and so on. A combination of two or more of these factors may exist.

The deeper pigmentation may be due to increased excretion of urochrome, or of urochrome plus one or more of the other three true urinary pigments, *viz.*, urobilin, porphyrin and uroerythrin.

Thus, in severe liver diseases (carcinoma, cirrhosis, lead poisoning), the excretion of all four may be increased. Fresh normal urine shows no absorption bands on direct spectroscopical examination, but in any condition in which urobilinuria occurs the band of urobilin may be visible either in the fresh urine or after treatment with hydrochloric acid (see Chapter XII). Urochrome gives no absorption bands. Porphyrinuria has been discussed in Chapter X. In "concentrated urines" it rarely, if ever, happens that the porphyrin is in sufficient concentration to yield absorption bands, and even in severe liver disease it is unusual to observe the absorption bands of (alkaline) porphyrin on direct examination. Uroerythrin is often visible in concentrated urines, since it stains the amorphous urates with which it is deposited pink ("brick-dust deposit"). If such a deposit is filtered, the precipitate on the filter paper turns green on making it alkaline by adding a few drops of sodium hydroxide solution (10 to 40 per cent). The pigment is readily extracted by amyl alcohol, and the solution exhibits two rather indefinite absorption bands (Fig 48). Uroerythrin in suspension in urine, or in pink urate sediments, gives a single band in the green close to the D line. Considerable quantities of uroerythrin are often excreted in gross liver diseases (new growth, cirrhosis, lead poisoning, etc.), and this pigment is often responsible for the deep reddish orange colour of highly pigmented pathological urines.

Garrod (1897) says that it is often possible to recognise the superimposed spectra of urobilin, porphyrin (alkaline) and uroerythrin in amyl alcohol extracts of highly coloured urines. A few hours of exposure to bright daylight suffices to remove both the colour and the band of uroerythrin, wherefore the bands of the other two pigments become clearer. If a few drops of concentrated hydrochloric acid are then added, the spectrum of alkaline porphyrin changes to that of acid porphyrin, leaving the urobilin band unaltered.

Nearly all urines become more pigmented and generally turn reddish when treated with mineral acid (e.g., about 1 c.c. of concentrated HCl to about 5 c.c. of urine), and particularly if warmed. This change in colour is due to the formation of pigments from chromogens. Several examples have already been given (e.g., urobilinogen to urobilin), but probably the most potent cause of the colour intensification and especially of the development of a reddish tint is the formation of urorosein from its chromogen. In Plummer's *Practical Organic and Biochemistry* it is stated that urorosein is probably nitrosoindole acetic acid, that fresh urines require the addition of a little nitrite solution as well as HCl, but that stale urines give the reaction with HCl only. This pigment is not extracted from acid urine by chloroform, whereas indigo blue and indigo red are, all three are extracted from acid urine by amyl alcohol. Urorosein shows an absorption band in the green (Fig 48). Its chromogen is probably of intestinal origin, and is possibly indole-acetic acid, as implied above.

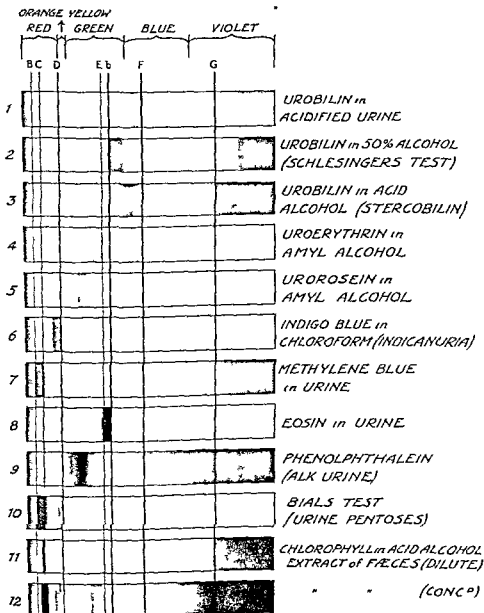


FIG 48 Absorption spectra of pigments in urine etc

In infants and children the urine may become abnormal in colour (bright yellow, pink, red, green etc.) as a result of sucking or chewing clothes, toys, water colour paints and so on. Thus Faught has reported a case of greenish blue urine, due to chewing a blue blotter (probably dyed with methylene blue).

The brownish black urine of *alkaptonurics* has been described in Chapter VI, urine may acquire a bluish tint from indigo-blue in *indicanuria* (see Chapters IV and XIV).

### Melanogenuria and Melanuria

When there are extensive secondary deposits of melanotic sarcoma in the liver, melanogen, the colourless chromogen of melanin, is excreted in the urine. The primary melanotic growth may be situated in the eye or in the skin, or occasionally in the internal organs. Melanogenuria, however, does not occur, or, at any rate, cannot readily be detected in the early primary stages of the disease (cf Garrod, 1902). The interval between the surgical removal of the primary focus and the secondary involvement of the liver may be a long one. Intervals of a few months up to twenty years have been reported. For this reason, as pointed out to me by Dr G. Graham, the urine of a patient who has had an eye removed, and who has an enlarged liver, should always be tested for melanogen.

Cases have been reported (cf Haden and Orr, and Peters) in which there is melanogen in the urine, but no melanotic growth in the body, but these are very rare and in not a few it is doubtful if melanogen was really present. If clinically there is evidence of a primary malignant growth and enlargement of the liver is found, the finding of melanogenuria is diagnostic.

The fresh urine is usually normal in colour, at any rate, it is not dark, because melanogen and not melanin is excreted. On standing the urine darkens (from above downwards if undisturbed) owing to the oxidation by air of the melanogen to melanin. This oxidation occurs slowly, and the urine does not become dark until it has stood overnight, or for twenty four hours or longer. Hence, it often happens that the presence of melanogen is not suspected from the appearance of the urine. "Melanogenuria" is therefore strictly the better term, but "melanuria" has long been applied in clinical work.

There are two main groups of chemical tests for melanogen. In the first an oxidising agent is used which would change melanogen to melanin and the resultant darkening of the urine is taken as evidence of the presence of melanogen. In the second, use is made of the reducing action of melanogen. The oxidising agents commonly employed are ferric chloride, nitric acid, bromine water and potassium chlorate with HCl. Urine containing melanogen reduces sodium nitroprusside to ferric ferrocyanide (Thormahlen reaction), and may reduce ammoniacal silver nitrate in the cold. It may also give Ehrlich's reaction with *p*-dimethylaminobenzaldehyde, a red colour with sodium nitrite and HCl, and a diazo reaction (Eppinger).

Professor H S Raper, in a personal communication, informs me that the substance responsible for the ferric chloride reaction can be separated from the substance giving the Thormahlen (nitroprusside) reaction. This is interesting because it has been claimed (*cf* Von Jaksch) that urines not containing melanogen may very occasionally give a positive Thormahlen test. The writer has tested many pathological urines, but so far has never obtained a positive Thormahlen reaction except in melanogenuria. His present conclusion is that if both the ferric chloride test and the nitroprusside reaction are positive, it is safe to diagnose melanogenuria.

When the concentration of melanogen in the urine is considerable, all the above mentioned reactions will be obtained, and the diagnosis is certain. Indeed, in such cases, on boiling the urine with Benedict's reagent, a peculiar green black precipitate, or with Fehling's solution a grey black precipitate, may be obtained, and, when performing Rothera's test for acetone bodies, the urine may become blue black. These reactions with Benedict's, Fehling's and Rothera's reagents, however, only occur in the terminal stages of the disease, and when melanogenuria is intense.

When the concentration of melanogen is small, both the nitric acid and bromine water tests are negative, and the urine does not

Test.	Alkapton Urine	Urine Containing Melanogen
Exposure to air	Darkens slowly from above downwards	Darkens slowly from above downwards
Addition of excess of alkali (e.g. NaOH)	Darkens in few seconds	Does not darken appreciably
Addition of excess of acid (e.g. HCl)	No appreciable darkening	Darkens slowly
Boiled with Benedict's reagent	Turns greenish black precipitate at first dirty brown, later yellow	No change or green black precipitate (much melanogen)
Boiled with Fehling's reagent	Turns black precipitate at first greyish black later red	No change, or greyish-black precipitate (much melanogen)
Silver nitrate alone	Turns black in few seconds often with blue sheen (colloidal silver)	No change, or very slowly turns brown (much melanogen)
Ammoniacal silver nitrate	Turns black at once	No change or slowly turns brown and then black (much melanogen)
Ferric chloride	Transient green or blue with each drop. No darkening even with excess	Changes to brown or black. No transient green or blue
Bromine water	No change	No change, or gives grey black or black precipitate (much melanogen)
Nitric acid	No blackening	No change, or brown to black (much melanogen)
Thormahlen's nitroprusside reaction	Negative	Positive. Turns blue or green

reduce ammoniacal silver nitrate. The ferric chloride and Thormahlen reactions are the most sensitive and are recommended for routine use.

All the tests are for melanogen and not for melanin. If, therefore, the urine has been allowed to stand for a considerable time and the melanogen has all been oxidised, the tests will be negative.

Once the characteristic reactions have been observed, mistakes are not likely to be made, but the condition is rare. Darkening on addition of oxidising agents may occur if there is gross indicanuria, or if the urine contains an abundance of pigment (urobilin, uroerythrin, etc.) and chromogens as in advanced liver disease or if the urine of a patient who has been taking certain drugs (particularly phenyl derivatives) is examined. In these cases, however, the Thormahlen reaction will be negative.

Alkapton urine (Chapter VI) behaves in many ways similarly to urine containing melanogen, but the ferric chloride and Thormahlen reactions will readily differentiate the two conditions, as will be seen from the comparison of tests (Harrison) on p. 222.

Urine containing melanogen may be preserved for weeks or months by adding  $\text{H}_2\text{SO}_4$  (saturated  $\text{SO}_2$  solution) to the extent of 1 per cent (H. S. Raper, personal communication). The preservative does not interfere with the Thormahlen reaction.

Linnell and Raper have shown that the chromogen of melanuria is probably either an ethereal sulphate or a glucuronate of 5, 6 dihydroxyindole.

### Tests for Melanogen in Urine

**Ferric Chloride Reaction.** To about 5 c.c. (1 in. column) of urine in a test tube add 10 per cent ferric chloride drop by drop. At first a precipitate of phosphates will form, and this may carry down with it the melanin formed by oxidation of melanogen. The precipitate will therefore be brown, grey or black. On further addition of ferric chloride the phosphates will redissolve owing to the acidity of the reagent, leaving a slightly turbid mixture, which will be brown, brown black, or black, depending on the concentration of the melanin.

**Ferric Chloride in HCl Test.** To about 5 c.c. of urine add about 1 c.c. of 10 per cent ferric chloride in 10 per cent v/v HCl. The urine will turn brown, brown black or black. There will be no precipitate of phosphates. The colour is often darker than in the first test, and this modification is particularly useful when the quantity of melanogen is small.

**Thormählen's Nitroprusside Reaction.** Prepare a fresh solution of sodium nitroprusside by shaking up a few crystals in about 10 c.c. of water. To about 5 c.c. of urine add 3 or 4 drops of the nitroprusside solution, and 10 to 12 drops (0.5 c.c.) of 40 per cent sodium hydroxide until the mixture is strongly alkaline, and shake. Then add a few c.c. of 33 per cent acetic acid (without heating) so that the mixture is acid. If melanogen is present the mixture will become blue to blue black, depending on the concentration of the



**melanogen** The blue colour is due to the formation of Prussian blue. If the urine is highly pigmented, the colour may be deep green instead of blue.

The technique is essentially the same as in Legal's test for acetone and aceto acetic acid (p 175). On making alkaline with NaOH, acetone, aceto acetic acid and creatinine give a deep ruby red colour, but on acidification acetone and aceto acetic acid give a purple red, and creatinine a dirty brown colour which only becomes green on warming.

With a negative urine the final mixture is amber or pale brown, but may have a slight greenish tint which must not be confused with the deep green (or blue) of a positive reaction. It is wise to make control observations on normal urines if there is any doubt whether a patient's urine is positive.

**Reduction of Silver Nitrate in the Cold** To about 5 c.c. of 3 per cent silver nitrate add about 0.5 c.c. of urine, and 2 per cent v/v ammonia drop by drop till most of the precipitate redissolves. If the mixture darkens before the precipitate has redissolved, stop adding the ammonia solution.

Normal urine treated in this way will remain practically colourless, or will turn brownish. When melanogenuria is intense the mixture rather slowly turns brown and then black. When there is little melanogen the reaction is negative. Other substances besides melanogen (e.g., homogentisic acid) will reduce silver nitrate in the cold.

**Bromine Test.** To about 5 c.c. of urine add bromine water drop by drop. When there is much melanogen a black or grey black precipitate will form. If there is little melanogen, the precipitate (if any) will be white or brownish white, but will not darken.

**Nitric Acid Test** To about 5 c.c. of urine add a few drops of concentrated nitric acid. Either no appreciable darkening, or a deep brown, or a black colour, will result, depending on the amount of melanogen. The test is not so sensitive as the ferric chloride reaction.

**Helman's Test.** To make sure that the darkening with oxidising agents is due to melanin proceed as follows —

To 20 c.c. or more of urine add cautiously 10 per cent ferric chloride till the maximum precipitate has been obtained, and filter. Shake up the precipitate with 1 per cent sodium carbonate and filter. To the filtrate add 10 per cent v/v HCl till it is acid to litmus. A brown black or black precipitate is due to melanin. The test is positive when melanogenuria is marked, but the writer has found it negative in the early stages of melanogenuria due to proved melanotic sarcoma of the liver, at which period both the ferric chloride and Thormahlen reactions were positive. To insist therefore on the necessity of a positive Helman's test will lead to failure in diagnosing the slighter grades of melanogenuria.

Helman, however, in the publications to which reference is made, gives no details. The technique given above is the one the writer has employed, and it may not be the best. In precipitating the

phosphates with ferric chloride, great care has to be taken to avoid an excess of the reagent in which both phosphates and melanin are soluble. At the moment, therefore, the writer does not regard the test as of much value clinically, but a description of it has been given because in several articles stress has been laid upon the necessity of obtaining a positive reaction before diagnosing melanogenuria. Further investigation is required.

### DRUGS IN URINE

The chemical pathologist must be interested in drugs and their products in urine, because they may cause abnormal colours, or because they produce unusual reactions during his routine chemical examination or because he is specially requested to make tests for their presence in cases of suspected poisoning. The first and second reasons are in evidence almost daily, the third is much less common and leads to work which is more often undertaken by a toxicologist, or an analyst who specialises in these investigations. For this reason the briefest of references only is made to the last group, for, in the writer's opinion, it is best considered together with the examination of the other excreta and the viscera, a subject which requires a volume to itself.

Comparatively little is known of the exact form in which drugs and their products are excreted in human urine, and particularly with regard to the newer therapeutic substances, there is room for considerable investigation on these lines.

It is convenient for the present purpose to classify drugs in urine as follows —

(A) Drugs which may affect the colour of urine—

- (i) Abnormal colour only when reaction is alkaline
- (ii) Different colours at acid and at alkaline reaction
- (iii) Same abnormal colour at all natural reactions

(B) Drugs which indirectly may influence the colour of urine

(C) Drugs which do not influence the colour, but which may cause unusual responses during routine testing

(D) Other drugs and poisons

(A) Drugs which may Affect the Colour of the Urine

(i) *Reaction alkaline*, naturally or due to bacterial decomposition. This group includes rhubarb and senna (chrysophanic acid), and the indicators phenolphthalein ('purgin') and phenol red. In the case of rhubarb and senna the fresh acid urine may be normal or orange in colour, but on adding alkali, or if the urine becomes alkaline on standing, it turns a reddish-orange. The colour change is sometimes not very marked in which case the following may be more striking. Add 10 per cent NaOH until the urine is strongly alkaline and a precipitate of phosphates occurs. Filter. The precipitate is often orange red or purple red owing to the adsorption of chrysophanic acid. On adding a drop or two of concentrated HCl the precipitate turns yellow. The urine shows no absorption bands.

**Phenolphthalein** is a purgative as well as an indicator, and is excreted unchanged. In acid urine it is colourless. When the urine becomes strongly alkaline (on standing, or after adding NaOH, etc.) it imparts a red tinge to the specimen. Alkaline urines containing this drug are not infrequently sent for examination for blood. They may show an absorption band in the green just to the right of the D line (Fig. 48). The indicator is not extracted by amyl alcohol.

**Phenol red** is used as a test of renal efficiency (see Chapter V), and apart from this it rarely if ever appears in the urine. It is yellow in acid, red in alkaline urine, being excreted unaltered.

(ii) *An abnormal colour whatever the reaction of the urine, but the colour changes with change in reaction.* Thus **santonin** makes fresh acid urine greenish or intensely yellow, and the colour changes to pink or purple on adding an excess of alkali. **Santonin** is an anthelmintic. It is excreted as the sodium salt, or as **oxysantonin**, which give no absorption bands.

(iii) *Same abnormal colour at all natural reactions.* This group contains the majority of the drugs listed on pp. 217 and 218.

**Carboluria** is caused by taking carbolic acid by mouth, or by absorption through the skin when phenol is used as a dressing. There may not be any appreciable change in colour, or the urine may become greenish or brownish black. The green colour is due to oxidation products of hydroquinone (*p*-dihydroxybenzene) or of pyrocatechin (*o*-dihydroxybenzene) which are formed from the phenol. The brown colour presumably is due to melanins, or melanin like products, resulting from oxidation of the phenol. Carbolic acid is excreted as an ethereal sulphate or as a glucuronate. The urine may reduce Fehling's or Benedict's reagents (glucuronate), and with bromine water in excess it may yield a white or yellow precipitate of tribromophenol (bromine also precipitates proteins). Often, however, the carboluria is slight and the above tests negative or inconclusive, and it is best to distil the urine after making it strongly acid with 10 per cent *v/v*  $\text{H}_2\text{SO}_4$ . The distillate is then tested with bromine water, when a white precipitate is readily obtained. The untreated urine occasionally will give a fleeting purple colour on the addition drop by drop of 10 per cent ferric chloride (*cf.* preparation of Uffelmann's reagent, Chapter XXII). The same test may be applied to the distillate but in both cases the reaction is generally unsatisfactory. A positive result with  $\text{FeCl}_3$  is valuable, but a negative reaction is of no significance. In carboluria there is nothing characteristic on spectroscopical examination. Owing to the conjugation of the phenol, the ethereal sulphates increase at the expense of the inorganic sulphates (*cf.* p. 290). Prolonged treatment with carbolic acid for several years has occasionally produced ochronosis just as occurs late in alkaptonuria (*cf.* p. 120). In both conditions the brown pigmentation of the cartilages and ligaments is presumably due to the deposition of the melanin like oxidation products.

In **lysol-poisoning** the urine is brown or brownish black, and gives the same reactions as in carboluria, as would be expected.

because lysol contains cresols (hydroxytoluenes) in an alkaline soap solution, lysol B P (*Liquor Cresolis Saponatus*) contains about 50 per cent v/v cresols, but the public have usually purchased "lysol solution" or "diluted lysol," which is much less concentrated, in attempted suicide therefore varying concentrations of cresol may be swallowed, *e.g.*, 5 per cent

It is wise always to distil the urine after strongly acidifying with  $H_2SO_4$  and to test the distillate with  $FeCl_3$  solution (blue colour), and with bromine water (white precipitate of tri brom compound), because the original urine often fails to give clear cut reactions with these reagents. In two cases of lysol poisoning the writer observed that the brown urine turned green or blue on adding sulphosalicylic acid (as in testing for protein) or HCl, but not with acetic acid, this may be of assistance in the early recognition of lysol poisoning, it was not observed in the specimens of urine passed later than about twenty four hours after taking the lysol, although the brown colour persisted. Amyl alcohol extracts the brown pigment(s) from the untreated urine, and removes incompletely the blue pigment from the acidified urine, a more concentrated solution of the blue pigment is obtained by separating the amyl extract of the non acidified urine and shaking it with a drop of concentrated HCl.

Spectroscopically the amyl extracts of the untreated urine in the earliest stages showed three absorption bands  $\alpha$  in the red and narrow,  $\beta$  in the orange and narrow and  $\gamma$  wide and less defined extending from the centre of the green into the blue. In subsequent specimens first  $\alpha$  and then  $\beta$  disappeared  $\gamma$  remaining when  $\gamma$  only was left neither the urine nor the amyl extracts turned blue with acid. The acidified blue urine showed a band in the red. In the acid amyl extracts this band was widened and shifted a little towards the violet.

Phenylhydrazine, used in the treatment of polycythæmia, may cause both acid and alkaline urines to become brown presumably due to the excretion of oxidation products (? melanin like products). The brown pigment is extracted by amyl alcohol, especially after acidifying the urine with HCl. Spectroscopically there is an ill-defined absorption of the green. Poisoning by excessive administration of phenylhydrazine has led to methæmoglobinæmia (Chapter XVIII) and to methæmoglobinuria (Chapter X), in which case the drug causes browning of the urine both directly and indirectly.

Pyramidone, or amidopyrine (dimethylaminophenazone), as such, or in combination or in mixture with a number of other drugs sold under different proprietary names (*e.g.*, veramon), is of special medical interest at the moment in connection with agranulocytosis. It is excreted as a glucuronate, which may cause a slight reduction, and as rubazonic acid, a condensation product of pyramidone which is red. Acid and alkaline urines have a pink colour, or a tint like that of a blood-orange or are red, depending on the concentration of the rubazonic acid, the colour is often mistaken for that of blood. The red pigment is unstable becoming less marked when the specimen is kept for a day or more. Spectroscopically there is an

ill defined absorption of the green blue, but no band. An excess of either HCl or NaOH changes the colour to yellow. The red pigment is very readily extracted by amyl alcohol.

Pyridium (phenylazo  $\alpha\alpha'$  diamino pyridine hydrochloride) and Neotropin (2 butyloxy 2' 6' diamino 5 5' azopyridine) are both employed as urinary antiseptics. They are said to be excreted unchanged, and make the urine deep orange or orange red. Excess of NaOH causes no significant change in colour, excess of HCl makes the colour deeper and more red. Spectroscopically the untreated urine shows no definite band, but a very intense general absorption of about half of the green and of all the blue violet. In urine well acidified (with HCl or acetic acid) the absorption is shifted obviously towards the red, so that almost all the green may be absorbed. Amyl alcohol extracts the dye partially from both untreated and acidified urine.

Prontosil or Prontosil Red (4' sulphamido 2 4 diaminoazo benzene hydrochloride) for oral administration and Prontosil soluble or Streptozone S (disodium salt of 4' sulphamido phenyl 2 azo 7 acetyl amino 1 hydroxy naphthalene 3 6 disulphonic acid) for injection, are used in the treatment of streptococcal and meningococcal infections. They both make the urine red, and the red urines behave very similarly to the urine after pyridium or neotropin. Their bactericidal action is due to reduction to *p* aminobenzene sulphonamide (sulphanilamide)—*cf* Fuller—which is colourless. They are excreted in the urine, partly unchanged and partly as sulphanilamide.

Sulphanilamide itself is now used for treatment under various names such as Prontosil Album sulphonamide P but since it does not colour the urine it does not concern us here.

The presence of these drugs in urine may cause difficulty in the interpretation of the results of several routine tests (*e.g.* Fehling's, Rothera's and Schlesinger's and spectroscopical examination), and it may be wise to repeat such tests after stopping the administration of the dye.

*The administration of guaiacum may cause the urine to become green.* The green pigment tends to rise to the surface if the urine is left undisturbed, because the resin is light. It is extracted by amyl alcohol, but yields no absorption bands. Guaiacum is probably the cause of the green urine sometimes seen after taking Eade's pills (sodium salicylate, guaiacum, aloes and colchicum).

*Methylene blue may render the urine green, greenish blue or blue.* The colour is blue if the dye is deliberately added to urine. Small quantities of methylene blue may be excreted as a colourless chromogen which can be converted into the pigment by adding acetic acid and boiling. Urine containing methylene blue shows an absorption band in the red (Fig. 48). The dye is extracted by amyl alcohol and the absorption band in the extract is slightly shifted towards the violet. The colour is unaffected by adding 10 per cent HCl or dilute (*e.g.* 1 per cent) NaOH, but it is destroyed

or rather changed to a dirty yellow brown, by adding several drops of 40 per cent  $\text{NaOH}$  to about 5 c c of urine. Methylene blue may be reduced to the leuco base as follows. To about 5 c c (1 in column in a test tube) of urine add a large knife point of glucose powder, and 1 or 2 or 3 drops of 40 per cent sodium hydroxide, so that the mixture becomes strongly alkaline to litmus but does not lose its green colour. Boil well for several minutes if necessary. If the green colour is due to methylene blue, it will disappear, to reappear on cooling and shaking with air. The process may be repeated many times. So far as the writer is aware no other substance, causing the urine to be green, would give this test.

Methylene blue is used as an analgesic in rheumatism, migraine, neuralgia and sciatica. It has also been recommended in inflammations of the urinary passages and in malaria. It is a constituent of certain proprietary medicines (e.g., de Witt's pills). It may be absorbed through the chewing of clothes dyed with it, or through the eating of coloured sweets, and so on. Methylene blue has in the past been used as a test of kidney function, and of liver function (cf. Barton), but in both cases it has been discarded as unsatisfactory.

Indigo-carmine is used as a test of renal efficiency (see Chapter V), and apart from this it rarely if ever appears in the urine. It imparts a greenish blue tint if the urine is acid or slightly alkaline, but if the reaction is strongly alkaline the indigo carmine is decolourised. If there is marked pyuria the colour does not develop. The green urine does not give an absorption band in the red, on boiling about 5 c c of urine made alkaline with 1 or 2 drops of 40 per cent  $\text{NaOH}$  the green colour disappears, whether or not glucose is also added and does not reappear on shaking with air (distinctions from methylene blue).

Garrod (1897) has seen a rich green colour produced by indigo-blue in the urine of a child who had sucked a rug dyed with that substance.

The urine is not infrequently coloured by chemicals used as antiseptics when washing out the bladder. Flavine (acriflavine) which turns the urine lemon yellow with a green fluorescence, is given on p. 218 as an example.

**General Remarks.** It will be noted in the above that no specific or elaborate tests are given for the various drugs and their products which may alter the colour of the urine. The reason is that in clinical work the simple reactions noted, plus the observation of the effect of discontinuing the drug give all the information required.

It should be added that the drugs mentioned do not invariably affect the colour of the urine. Naturally the dose, the absorption of the drug, and other factors play their part, but in addition, clinically, the impression is gained that some patients appear to be more liable than others to excrete coloured products. This impression is strengthened by the frequent failure of the observer to reproduce the colour in his own urine by taking the drug responsible for an abnormal tint of a patient's urine.

**(B) Drugs which indirectly may Influence the Colour of Urine**

Examples of drugs in this group have already been given under the headings of methæmoglobinuria (Chapter X) caused by phenylhydrazine (see also p 227), and of porphyrinuria (Chapter X) caused by sulphonal, trional and veronal

**(C) Drugs which do not Influence the Colour of the Urine, but may cause Unusual Responses during Routine Testing**

The examination of urine for certain drugs is of clinical importance in the diagnosis of poisoning, of drug rashes, and so on, and in the recognition of some of the ingredients of medicines taken by patients when the prescriptions are not available. In the laboratory, when carrying out urinary examinations, it frequently happens that peculiar (colour) reactions due to drugs are observed, and it is important to be able to recognise these "false" reactions, and to make inquiries as to the nature of the drug treatment. Only drugs commonly employed will be mentioned.

**Bromides** are excreted as such. They may cause skin eruptions (bromism). They make the chloroform extract yellow brown when performing Jaffe's test for indican (Chapter XIV).

(a) To about 5 c.c. of urine add an equal volume of concentrated hydrochloric acid and about 3 c.c. of chloroform. Add 1 or 2 per cent potassium chlorate, a drop at a time, inverting the mixture repeatedly after each addition. If bromides are present they will be oxidised to bromine, which is extracted by the chloroform, to which it imparts a yellow brown tint. The reagents alone, however, will give a yellow colour owing to the formation of chlorine, which is extracted by the chloroform, so that it is advisable to treat similarly a normal urine at the same time, and to compare it with the urine suspected to contain bromide. The advantage of adding the potassium chlorate in portions is that it avoids the risk of over-oxidising the bromide.

(b) To about 10 c.c. of urine add 2 or 3 c.c. of 10 per cent  $\text{v/v}$   $\text{H}_2\text{SO}_4$  and a few drops of a saturated potassium permanganate solution till the mixture remains permanently pink. Warm and note the colour and smell of the vapour. Bromine has a brown colour and a characteristic smell, and makes the eyes water.

**Iodides** are excreted as such. They may cause skin eruptions (iodism). When they are present in the urine, on performing Jaffé's test for indican (Chapter XIV) the chloroform extract is purplish red.

(a) To about 5 c.c. of urine add an equal volume of concentrated hydrochloric acid and about 3 c.c. of chloroform. Add 1 or 2 per cent potassium chlorate, a drop at a time, inverting the mixture repeatedly after each addition. Iodides are oxidised to iodine, which is extracted by the chloroform which therefore becomes purplish red. On adding a few drops of 10 per cent sodium thiosulphate and again repeatedly inverting, the iodine is decolourised, thereby differentiating it from indigo red, which is

unaffected The addition of potassium chlorate in stages avoids the over oxidation of iodides to the colourless iodates

(b) To about 5 c c of urine add 1 or 2 c c of chlorine water and extract with chloroform, etc., as above

(c) To about 5 c c of urine add 1 or 2 c c of chlorine water and a few drops of starch solution (about 1 per cent) The iodides are oxidised to iodine, which turns blue with starch Instead of chlorine water, HCl and potassium chlorate may be used to liberate the iodine (*cf* under (a))

(d) To about 5 c c of urine add 2 or 3 c c of chloroform and concentrated nitric acid, a few drops at a time, inverting between each addition Then proceed as in (a) There is more risk of over oxidation with nitric acid than with procedure (a), which is recommended in preference to (b) (c) or (d)

Salicylates are excreted as salicylic acid or unchanged Salicylic acid is formed by the conjugation of salicylic acid with glycine, and is an example of synthesis by the kidneys It is a common cause of a slight reducing action of urine on Benedict's and Fehling's reagents (see Chapter VI), since aspirin (acetyl salicylic acid) is a drug which is very extensively employed Salicylates have also been mentioned in Chapter IX since urine containing them gives a purple colour with ferric chloride, which has often to be distinguished from the Bordeaux red of aceto acetic acid in Gerhardt's test Both acetyl salicylic acid and salol (phenyl salicylate) yield salicylic acid in the body, and are excreted as salicylic acid or as salicylates

(a) Boil the urine with Benedict's or Fehling's solution (see Chapter II)

(b) To about 5 c c of urine add 10 per cent ferric chloride drop by drop At first a white precipitate of phosphates is obtained Then the urine turns purple or Bordeaux red if salicylates are present

Boil thoroughly about 10 c c of urine in a boiling tube, cool, and add the ferric chloride If the urine contained salicylates it will still give the purple colour, if it contained only aceto acetic acid there will be no colour change, if it contained both substances the purple colour will be less intense than before In either of the last two events confirm the presence of aceto acetic acid by Rothera's test (see Chapter II)

(c) To about 5 c c of urine add ferric chloride as before If a purple colour results, add a few drops of acetic acid when the colour is not discharged Then add a few drops of sulphuric acid (20 to 50 per cent), when the purple will be destroyed

(d) To about 5 c c of urine add an excess of bromine water Salicylates, like phenol, give a white or yellowish precipitate of the tri brom compound A distinction may be made between the two substances by distilling the acidified urine (see under Carboluria) Phenol comes over readily, salicylic acid very slowly and only in traces

Phenazone (antipyrine) is excreted as an ethereal sulphate, or as



a glycuronate which may cause a slight reduction. The urine is occasionally red. With ferric chloride the urine becomes Bordeaux-red, which may be distinguished from the colour due to acetoacetic acid by boiling, as described for salicylates under (b) (Cf also Chapter IX.)

**Uroselectan** (sodium salt of 5-iodo-2-pyridone N-acetic acid) is used for making the urine opaque to X-rays, thus enabling a radiogram of the urinary tract to be obtained. A large dose (up to 40 gm) is injected intravenously, of which 80 to 90 per cent is excreted by healthy kidneys in about six hours. When urine containing uroselectan is acidified with a strong acid, a white crystalline precipitate is formed which may be copious. This may easily lead to erroneous conclusions when testing urine for protein with either nitric acid or sulphosalicylic acid, but uroselectan gives no precipitate in the boiling and acetic acid test. Fortunately differentiation is simple, protein is insoluble in alcohol, whereas the precipitate from uroselectan is readily dissolved on adding about 2 volumes of absolute alcohol and warming. On adding a few drops of 10 per cent ferric chloride to the urine an orange or yellow-brown precipitate is formed. Otherwise this drug gives no peculiar reactions during routine urinary testing.

The reader is referred to Chapter VI for a list of the various drugs which are conjugated with glycuronic acid, and which therefore may cause a slight reduction of Benedict's or Fehling's solution.

#### (D) Other Drugs and Poisons

The chemical pathologist is occasionally asked to examine urine for metals, for derivatives of barbituric acid, and for other drugs which, taken in excess of the therapeutic doses, are poisonous. In addition, his services may be requested in cases of industrial poisoning. For the reasons given on p. 225, a consideration of this subject is deliberately omitted, except for the few points of clinical importance in chemical tests for lead and arsenic given below.

In lead poisoning (neuritis, wrist drop, colic, encephalopathy, etc.) more of the metal is excreted in the faeces than in the urine. *Both excretions should be sent for analysis, 500 c.c. of urine or more are required.* The finding of lead is of great value, but a negative result does not exclude the diagnosis, since no appreciable quantity of the metal may be excreted at the time of the test. The quantity excreted in the urine in twenty-four hours varies from 0 to about 10 mgm of metal. The characteristic blue line on the gums is due to the deposition of lead sulphide. The urine in acute cases is very deeply pigmented, and, as mentioned previously, contains a great excess of all four urinary pigments, viz., urochrome, urobilin, uroerythrin, and porphyrin. In chronic poisoning considerable quantities of lead may be stored in the bones, probably as phosphate.

Traces of lead are found in the excreta in health, and it is fairly generally agreed that amounts up to 0.1 mgm Pb per 1,000 c.c. of

urine, and up to 50 mgm per 100 gm of dried feces are within normal limits (cf Davidson, *et al*)

Considerable attention has recently been paid to lead poisoning, apart from the ever present industrial poisoning, in view of the lead treatment of cancer and of the introduction of lead tetraethyl as an anti knock agent in motor fuel. A good review of the subject is given by Aub, *et al*, including methods. In clinical work the writer has found Thresh's method convenient.

In arsenic poisoning (neuritis, dermatitis, pigmentation, etc) the urine, hair and nails are usually selected for chemical analysis, though the metal is also excreted in the feces. For test purposes 100 c c or more of urine, and 0.5 gm or more of hair or nail parings should be supplied. A negative chemical finding does not exclude the diagnosis. In clinical work the Gutzeit test, as laid down in the British Pharmacopœia is convenient. Disappearance of arsenic from the hair requires a very long time, and the metal may be detected therein years after its intake has ceased.

In addition to industrial poisoning, the clinician is interested in arsenic poisoning resulting from excessive medication, and particularly from the use of salvarsan and its derivatives. Patients show considerable variation in their liability to poisoning by arsenic (and other metals), and before treatment with its compounds a careful clinical examination should always be made, particular attention being paid to the condition of the liver and of the urine (proteinuria, etc).

### A SIMPLE SCHEME FOR THE CLINICAL EXAMINATION OF URINES ABNORMAL IN COLOUR, OR CONTAINING DRUGS AND THEIR PRODUCTS

(i) Note the colour of the urine, and follow up any clues obtained from the presence of abnormal colours

(ii) Take the reaction to litmus

(iii) Note the effect of adding acid (HCl) and alkali (NaOH) respectively on the colour of the urine

(iv) Examine with the spectroscope (see Chapter X) —

(a) The untreated urine

(b) The same after addition of acid and of alkali in (iii)

(c) An amyl alcohol extract of the urine (Take about 10 c c of urine in a centrifuge tube. Add 2 or 3 c c of amyl alcohol. Shake well. Centrifuge and separate the amyl extract.)

(d) Repeat (c) after making the urine acid with HCl, and alkaline with NaOH respectively

(v) Note the effect of adding ferric chloride solution (Alkaptonuria, melanogenuria, salicylates, phenazone, carbolic acid)

(vi) Note the effect of treatment with oxidising agents (HCl plus  $\text{KClO}_3$ ) (Melanogenuria, indicanuria, indigo red, iodides, bromides)

(vii) Boil the urine with Benedict's or Fehling's reagent, and note if any reduction occurs (Alkaptonuria, melanogenuria, salicyluric acid, glycuronates.)

(viii) Note if a precipitate is given with an excess of bromine water (Melanogenuria, carbohic acid, salicylates)

(ix) Apply other tests indicated in this chapter, if a clue to the nature of the substance has been obtained from Nos (i) to (viii)

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## CHAPTER XII

### BILE AND UROBILIN IN THE URINE: EFFICIENCY TESTS OF THE LIVER AND BILE PASSAGES

**Books and Reviews** Rolleston and McNee's *Diseases of the Liver, Gall Bladder and Bile Ducts*, which in addition to being an exhaustive treatise, gives extensive references

Weiss's *Diseases of the Liver, Gall Bladder, Ducts and Pancreas*

Graham, Cole, Copher and Moore's *Diseases of the Gall Bladder and Bile Ducts*

Lepehne's *Die Leberfunktionsprüfung, ihre Ergebnisse, und ihre Methodik* Also *Munch med Woch*, 1922, 69, 342, and *Klin Woch* 1924, 3, 73

*Liver Function Tests* Mann, F C, and Bollman, J L, *Arch Path*, 1926, 1, 681 An excellent review of the possible clinical value of liver function tests judging from experimental findings

Roger's *Physiologie Normale et Pathologique du Foie*

*Diseases of the Liver* (Function tests, general survey, findings in different diseases and in experimental obstruction) Greene, C H, Rowntree, L G, Snell, A M, Walters, W, *et al*, Series of Papers in *Arch Int Med*, 1925, 36, 248, 273, 418 and 542, 1926, 38, 167, 1927, 40, 159 and 471

*The Extra Hepatic Functions of Bile* Schmidt, C L A, *Physiol Rev*, 1927, 7, 129

IN Chapter X, hæmoglobin and its derivatives in the urine have been reviewed In Chapter XI, the other substances (including drugs) which may cause the urine to be abnormal in colour have been discussed, with the exception of bilirubin and its derivatives, which will now be considered The subject of bilirubinuria leads naturally to the broader problem of tests of hepatic efficiency.

### BILIRUBINURIA

The urine is tested for bile pigments either on account of its colour, or of the colour of the patient's skin or conjunctiva, or as part of a routine examination Normally there is no bilirubin in the urine, or possibly the merest trace (*cf* Naumann) In general terms bilirubinuria signifies disease of the liver, obstruction of the bile passages, or excessive hæmolysis The tests for bilirubin have been given in Chapter II They depend on the oxidation of bilirubin to green (biliverdin) or blue (bilocyanin or cholecyanin) pigments, or on the formation of azobilirubin If oxidation is allowed to proceed too far a yellow pigment (choletelin) is formed, the colour of which is obviously of no help in urine Van den Bergh's diazo test for bilirubin in the blood (see later) can be applied to urine if the bilirubin is first separated by adsorption on a barium precipitate

(Hunter)—see p 19 Spectroscopically bilirubin gives no absorption bands, but merely a general absorption of light mainly at the violet end of the spectrum Cholecyanin does give absorption bands which vary with the reaction and the purity of the pigment in solution In clinical work, therefore, these spectra are seldom of much assistance, wherefore the reader is referred to larger works Biliverdin gives no absorption bands, whereas choletelin in acid solution gives a band between *b* and *F*, which, however, is not of much help in examining treated urine containing a mixture of oxidation products

### UROBILINOGENURIA AND UROBILINURIA

The urine is examined for urobilin on account of its colour, or during routine examination, or because liver damage or excessive hæmolytic or pernicious anæmia is diagnosed Normally urine contains traces only of urobilin or its chromogen, and these quantities do not affect the colour Where there is an excess of the pigment the urine is more orange than usual, and a pinkish yellow tinge may be noted in the lowest layers in a conical receiver Tests for the pigment plus its chromogen have been given in Chapter II These depend on the characteristic absorption spectrum (Fig 18), the fluorescence of the zinc urobilin compound, and the colour of the copper urobilin compound in a chloroform extract It is advisable also to examine spectroscopically the solutions obtained in the last two tests, because the fluorescence or the colour may be masked by other pigments, especially by bilirubin In clinical work there is usually nothing to be gained from tests which respond solely to the pigment or solely to the chromogen respectively, since both have the same significance, and since the chromogen rapidly changes into the pigment on exposing the urine to light and air Thus urobilinogen, but not urobilin, gives Ehrlich's reaction with para dimethyl aminobenzaldehyde, and this has been utilised by Wallace and Diamond in a method which gives approximately quantitative measurements of the concentration of the chromogen in urine The reader is referred to the articles of Terwen and of Watson for a discussion of the estimation and preparation of urobilin and its chromogen from urine and fæces

The relationship of urobilinogen and of urobilin to bilirubin is discussed more fully in the next section Urobilinogen is formed by the action of bacteria on bilirubin in the large intestine Normal urine in the fresh state probably contains only the chromogen In pathological conditions the fresh urine contains more chromogen than pigment, but when urobilinuria is marked, probably some fully formed pigment is excreted as such It is obvious therefore, that urobilinogenuria is really the more descriptive term but urobilinuria is generally used to include the presence of the pigment or chromogen or both, and it will be used in that sense here

In clinical work, whilst in general terms it may be stated that urobilinuria signifies either disease of the liver or excessive

hæmolysis, it is quite common to find urobilin in the urine of patients with all sorts of diseases not usually associated with gross liver disease. Thus urobilinuria occurs frequently in almost all fevers, but it then generally lasts only for a few days. Slight temporary urobilinuria may also be caused by simple constipation due to the increased opportunity of absorption from the large intestine.

In liver diseases (cirrhosis, malignant disease, passive congestion, etc.) urobilinuria is more persistent, though not invariably present. In the milder forms there is an increased excretion of urobilin without any conspicuous increase in the output of other pigments. In severe liver disease (advanced carcinoma, lead poisoning, etc.) there is a general increase of all pigments (cf Chapter XI).

In pernicious anæmia urobilinuria is a common finding, but is often intermittent, apart from the remissions when it is absent. In secondary anæmia urobilinuria is generally absent, though it may occur during a stage of very active hæmolysis. The finding of the pigment in the urine is, therefore, of limited value in differentiating between the two types of anæmia. Urobilinuria occasionally results from the absorption of a large extravasation of blood. For a fuller account the reader is referred to the publications of Garrod quoted in Chapter XI.

### THE CYCLE OF BILIRUBIN

The normal cycle of bilirubin is represented diagrammatically in Fig. 49. It will be seen that the greater portion is lost in the fæces mainly as stercobilin and to a lesser extent as stercobilinogen. A small proportion of stercobilinogen is absorbed into the blood. Of this the greater part passes back to the liver, completing the cycle of bilirubin. The liver either re-excretes as such this stercobilinogen, or oxidises it back to bilirubin which passes into the bile. A smaller portion of the stercobilinogen absorbed from the intestine escapes the liver, and passes into the general circulation, from which it is excreted by the kidneys as urobilinogen. Thus the normal trace of urobilinogen in the urine is explained.

The reduced bilirubin is termed stercobilinogen or hydrobilirubinogen in the fæces, and urobilinogen in the urine, these are probably merely different names for the same chromogen. Likewise urobilin, stercobilin and hydrobilirubin may be regarded as convenient terms for one and the same pigment.

In a similar way the bilirubin which is passing in the blood from different parts of the reticulo endothelial system to the liver might be called "hæmobilirubin," and that in the bile on its way from the liver to the duodenum "cholebilirubin." The significance of this suggestion will be clear later.

In pathological conditions this cycle of bilirubin and its derivatives may be upset in a number of ways, as will be seen by studying Fig. 49. Pathological states of the liver and bile passages will be discussed later in this chapter.

If there is excessive formation of hæmoglobin, or excessive hæmolysis, the quantity of bilirubin reaching the liver, and therefore

## THE NORMAL CYCLE OF BILIRUBIN AND ITS DERIVATIVES

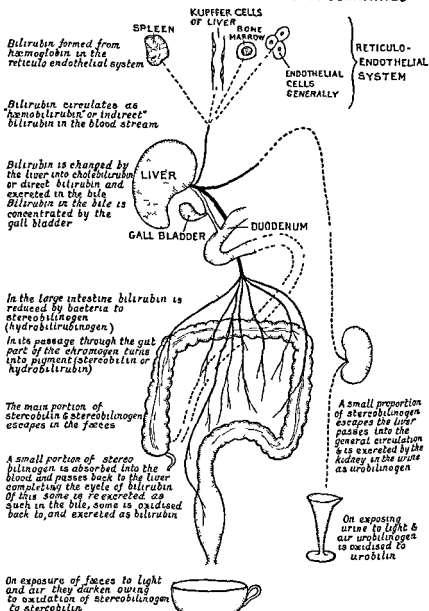


FIG 49.

Note "Non direct," as used in the text, is a better alternative for hæmobilirubin than is 'indirect' bilirubin

the amount of stercobilinogen formed in the colon, and therefore the amount of stercobilinogen absorbed into the blood, and therefore the amount of urobilinogen excreted in the urine, will be increased. This is one way of explaining the pathological urobilinogenuria in pernicious anæmia, or from the absorption of large extravasations of blood. The urobilinogenuria is accompanied by an excessive

excretion of stercobilinogen and stercobilin in the faeces, which therefore are dark

In constipation there is an increased opportunity of absorption of stercobilinogen into the blood, and therefore of the amount escaping the liver, hence slight urobilinogenuria occurs

### BILE SALTS IN THE URINE

Tests for bile salts (sodium glycocholate and taurocholate) are seldom made unless bilirubinuria also is suspected. They should be, however, because in certain stages of liver disease bile salts may be excreted unaccompanied by bile pigment—one type of so called “dissociated jaundice” (In the other type, bilirubinuria is unaccompanied by bile salts, *e.g.*, in uncomplicated hæmolytic icterus). Thus in catarrhal jaundice it has been stated that bile salts may appear in the urine before bilirubin, that with the development of bilirubinuria the bile salts may disappear, to reappear again for a few days as the urine becomes free from bile pigment. Gerrard has shown that bile salts may persist in the urine long after bilirubin has disappeared in cases of salvarsan jaundice. Comparatively little work, however, on bile salts in the urine in clinical cases has been done. It would obviously be worth while in patients treated with drugs which are potential liver poisons (*e.g.*, salvarsan and derivatives, lead, and so on), in order to try to obtain early warning of toxic action. Again, in patients with subicteric tints and fever of uncertain origin, the test might prove of clinical value. Indeed there is an obvious field for investigation both of normal and pathological subjects.

The chief difficulty is a technical one. In clinical work either Hay's test (see Chapter II) or Pettenkofer's reaction is generally employed. Hay's is a test for a lowered surface tension of the urine, and therefore is not specific for bile salts. Pettenkofer's reaction is performed as follows—

To about 5 c.c. of urine add 5 drops of 5 per cent. sucrose. Hold the test tube at an angle and carefully pour down the side 2 to 3 c.c. of concentrated sulphuric acid. A red ring indicates the presence of bile acids. On shaking the tube gently the whole layer of urine turns red. The tube should be placed under running water during shaking, because the red compound is destroyed if the temperature rises above about 70° C. The red solution gives two absorption bands, one at F, and the other between D and E, nearer E. The reaction depends on the formation of furfural by the action of the acid on sucrose. It is not a specific test for bile salts, and difficulties may be encountered if the urine contains protein or chromogens which yield interfering colours with sulphuric acid. Rolleston and McNee state that a dilution of 1 in 1,000 of bile salts gives a poor Pettenkofer reaction, whereas a dilution of 1 in 40,000 gives a positive Hay's test.

The ordinary ward procedure for Hay's test, using a 6 in.  $\times$   $\frac{5}{8}$  in. test tube, is given in Chapter II. McNee and Pritchard (see Rolleston and McNee's book), and Gerrard, point out that this



method is crude, and make the following recommendations. The urine should be fresh, clear, and cooled to room temperature. The flowers of sulphur should be dry, and therefore in the form of a fine powder, and should be sprinkled on the urine without touching the sides of the vessel, which must be wide (a small beaker, square glass cell, or conical receiver). If a beam of light is reflected from one side, even the scantiest fall of sulphur particles may be detected. The sulphur should be delivered from a standard height above the urine. McNee and Pritchard used an ordinary tin pepper box, clamped 25 cm (1 in) above the surface of the urine, which was tapped with the finger to eject the sulphur. Gerrard used a metal sprinkler perforated in the centre only, held  $\frac{1}{4}$  in above the urine. When the urine contained 1 in 10,000 of bile salt the sulphur sank instantly, when the concentration was 1 in 40,000 the sulphur rested on the surface for a minute or two before sinking. Very large doses of sandalwood, copaiba, cubebs, hexyl resorcinol and turpentine lower the surface tension, but chloroform, chloral, salicylates and iodides do not. Urines preserved with thymol may give a positive test. It has been stated that substances lowering the surface tension may occur in certain cases of nephritis.

Bile salts are absent from normal urine, or, at any rate, they cannot be detected by the above tests. Their presence in the urine can, with the possible exception of nephritis, always be regarded as indicative of liver damage or duct obstruction.

### THE CYCLE OF BILE SALTS

Bile salts are almost certainly formed in the liver. They are excreted in the bile and pass into the intestine. Normally none can be detected in the faeces or the urine, so there is no appreciable loss in the excreta. It has been shown that they are rapidly absorbed from the intestine into the portal blood to be picked out by the liver and re-excreted in the bile, thus completing the cycle. That they must be formed within the body is shown by the fact that they are excreted for a considerable period through a biliary fistula during starvation. But beyond these facts very little is known. If normally bile salts are being continuously formed endogenously, and *practically all the amount excreted is reabsorbed*, it is obvious that they would accumulate. Either their endogenous formation is not called into play until there is a loss from the body, or in some way or another they are continually being destroyed and new formation keeps pace with destruction. The work of Whipple *et al* points to protein (body protein or food protein) products as the source of bile salts, and not cholesterol or lipoids. In health the peripheral blood contains small quantities of bile salts. Since they are absent from the urine they must be 'threshold substances'.

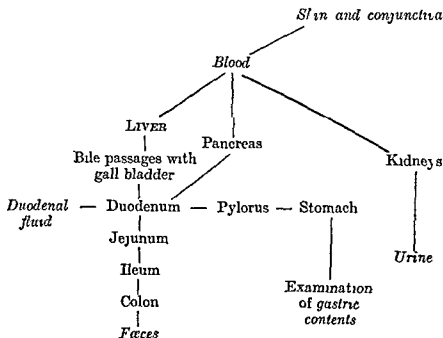
In pathological conditions, again, our knowledge is very limited. In the early stages of obstruction to the bile passages the amount in the blood increases, and bile salts appear in the urine. But with continuation of the obstruction they disappear from the urine, and often reappear for a short time when the obstruction is relieved.

These are the observed facts, and it is idle to speculate as to their meaning until more is known of the physiology of the subject

### CHEMICAL TESTS OF THE EFFICIENCY OF THE LIVER AND BILE PASSAGES

A number of chemical tests of function have been introduced as an extension of the clinical examination of patients who are suspected to have liver disease. In another group of cases there is no doubt clinically of the existence of liver damage, but the same tests are employed in an attempt to gain some idea of the efficiency of the liver remnant, or as a guide to progress of the liver condition, or as an aid to treatment. In some cases the simple routine urine tests for bilirubin urobilin or bile salts lead to the request for efficiency tests. In other cases these urine tests give no indication of the damage to the liver or bile passages which is evident from the clinical examination.

In general the same difficulties apply for the liver as for the kidney (cf Chapter V) when attempting to assess functional efficiency. Thus no test has been devised for detecting slight degrees of damage. Abnormal results are obtained only when the disease is very advanced. It is impossible to assess the 'reserve power'. The tests give little or no indication of the cause of the damage, but merely a rough measure of its degree. The possible influence of non hepatic factors on the tests is great, and must always be considered. Repeated observations are often necessary as a guide to progress and to treatment. But in the case of the liver the difficulties are greater than in the case of the kidney. Thus its



excretion is not so accessible. In fact, it is difficult if not impossible, to obtain pure human bile *in vivo*, apart from occasional opportunities at operation. The liver has even more functions than the kidney, and, as in the case of the kidney, one function may be more impaired than another in disease. Hence tests of several functions are generally desirable. Lastly, it is often particularly difficult to exclude the possible influence of the pancreas on tests of hepatic function.

The diagram on p. 241 represents the relationships of the liver and bile passages to other organs and body fluids, and enables the reader to see at a glance what material is available for examination when carrying out function tests.

### THE FUNCTIONS OF THE LIVER

The formation and excretion of bile

Metabolic functions in relation to carbohydrates, proteins and fats

The antitoxic power of the liver

Hæmopoietic functions

Excretion of foreign substances

It is impossible to consider the functions of the liver without simultaneously discussing those of the gall bladder and bile passages. Obstruction of these passages not only causes chemical changes in the body fluids, but also may cause damage to the liver itself. The main function of the gall bladder is the storage and concentration of the bile. This concentration is about sixfold to tenfold. Advantage of this concentrating function is taken in **cholecystography**. Suitable salts (tetrabromphenolphthalein, tetraiodophenolphthalein, or phenoltetraiodophthalein) are administered intravenously or orally. These are concentrated with the bile in the gall bladder wherefore an opacity is obtained on radiological examination. This is not a chemical test, so the reader is referred to other works.

### THE FORMATION AND EXCRETION OF BILE

Judging from the output of fistula bile the quantity normally excreted into the intestine is about 600 to 700 c.c. in the twenty-four hours (*cf.* Fox). The chemical composition of bile from the liver and of bile from the gall bladder is given on p. 243, the difference between the two sets of analyses being due to the concentration effected by the gall bladder. Comparatively few analyses have been recorded, and the range of variation in the results is tremendous. A series of complete analyses, using modern methods, is badly needed.

So far as the excretion of bile is concerned, efficiency tests are limited to bile pigments, bile salts and cholesterol. Reference to the diagram given above will show that theoretically in each case examinations might be made of the blood, urine, duodenal contents

stomach contents when regurgitation occurs, and faeces In jaundice the skin and conjunctiva are stained by bilirubin

*Approximate Percentage Composition of Human Bile*

Constituent	Gall bladder bile	Liver bile or Fistula bile
Bile salts	6.0	0.72
Mucus and bile pigments	3.0	0.40
Cholesterol	0.38	0.06
Fat, including lecithin and fatty acids, free and combined	0.82	0.07
Inorganic salts	0.80	0.75
Total solids	11.0	2.0
Water	89.0	98.0

*Notes* The figures for bile salts, cholesterol and total solids are the averages worked out by Fox from a study of the literature. He gives the ranges only for cholesterol which are as follows: Bladder bile 0.01 to 1.30; fistula bile 0.004 to 0.150 per cent. So many different methods have been employed and the records are scattered over so many years that little of value would result from working out the ranges for other constituents.

### Bile Pigment and its Derivatives

Examination of the urine and the cycle of bilirubin have already been discussed. Except in the case of gross diarrhoea, bilirubin is said never to be excreted in the faeces of adults, but is present in the stools of very young infants (*cf* p. 459). Further investigation of the dark faeces in acholuric jaundice and pernicious anaemia is required, however, for in some cases it has been found in the writer's laboratory that the excessive amounts of stercobilin and its chromogen are accompanied by small quantities of bilirubin. Biliverdin is excreted in meconium, and is responsible for the green colour. Otherwise bilirubin is always reduced in the large intestine and excreted as stercobilinogen and stercobilin. Examination of the faeces for the last two (see Chapter XXIV) is useful in the diagnosis of obstruction and in watching the progress of a case of jaundice. It has been stated that when the obstruction is complete the faeces are devoid of stercobilin and its chromogen, but, in common with several other observers, the writer has never failed to find slight traces of the pigment or its chromogen, even in proved cases of complete obstruction. The probable explanation of this is that bilirubin passes from the blood of these severely jaundiced patients into the bowel, where it is reduced by bacteria in the usual way. Those who hold that the reduced bilirubin is completely absent have probably used less sensitive methods, or have failed to examine for the chromogen. In clinical work the finding of mere traces

of stercobilin and stercobilinogen is a point strongly in favour of gross obstruction. In catarrhal jaundice and in toxic hepatitis daily fecal tests will frequently enable the return of bile into the intestine to be detected several days before there is any obvious improvement in the jaundice. Methods for measuring approximately the concentration and output of stercobilin plus stercobilinogen have been devised (*cf* Wilbur and Addis), but are of doubtful value in clinical work owing to the great variations in different normal individuals, and in the same individual on different days.

Examination of the gastric contents or vomit is of no value, beyond the fact that the presence of bilirubin obviously excludes complete obstruction of the bile passages. Examination of the duodenal contents is discussed in Chapter XXIII. It is of limited value.

Examinations of the urine and of the blood are the most important. There are three methods of testing the blood (for technique see end of this chapter).

**Fouchet's test** is useful in clinical work. It always gives positive results when the bilirubin content is 1 in 60,000 (*i.e.*, 3.3 Van den Bergh units, or 1.7 mgm of bilirubin per 100 c.c.), or more. At times it will detect less, some sera give positive results when the bilirubin is only 1 mgm per 100 c.c. The normal serum bilirubin is 0.1 to 0.5 mgm. Therefore if Fouchet's test is positive, bilirubin is present in pathological amount. On the other hand, the test is not sufficiently sensitive invariably to reveal slight hyperbilirubinæmia, such as is often found in pernicious anæmia.

**Meulengracht's test (Icterus Index or Bilirubin Index)** is a measure of the intensity of the yellow colour of the serum. In most, but not all cases, it is fair to assume that the yellow is mainly due to bilirubin (*cf* Chapter XVIII). The chief practical difficulty is to avoid hæmolysis during collection of the blood. It is generally agreed that an index above 6 indicates hyperbilirubinæmia. Most workers consider that normally the index may be as low as 1, but Bernheim regards any figure less than 4 as constituting hypobilirubinæmia. Förster, using this method, gives the normal range of bilirubin as 0.24 to 0.97 mgm per 100 c.c. of serum correspondingly approximately to an index of 1 to 5. In latent jaundice the icterus index usually lies between 6 and 15. The chief value of the test is in hypobilirubinæmia, in which quantitative measurements by Van den Bergh's method are difficult or impossible. At the same time, the significance of hypobilirubinæmia clinically has not been established. In the writer's experience it is extremely common to find little or no bilirubin in the sera of infants and children. In clinical work, therefore, the icterus index rarely, if ever, gives more information of value than Van den Bergh's test, and in hyperbilirubinæmia it certainly gives less. The writer has, therefore, practically discarded it except in carotinæmia, and when there is a suspicion that the yellow colour of a serum is not mainly due to bilirubin. In the latter even the combination of Meulengracht's and Van den Bergh's tests is often useful.

In Van den Bergh's test there are two distinct processes. In the first, known as the "direct reaction," the diazo reagent is added directly to serum or plasma, and any colour change and the time taken for its development is noted. In the second the serum is treated with the diazo reagent, and the proteins are precipitated with alcohol, which also extracts the azobilirubin. The intensity of the resulting purplish red is measured quantitatively.

Normal sera give a negative direct reaction in the sense that no colour appears within about thirty minutes after adding the reagent. Some normal sera do show a reddish tint if the mixture is observed for longer periods, *e.g.*, one hour, this can clearly be demonstrated by the simultaneous preparation of a comparison tube containing serum, but water instead of the diazo solution.

Pathological sera containing excess bilirubin may or may not give a positive direct reaction. Those giving a negative response were said to contain the excess of bilirubin in the 'non direct' form or haemobilirubin. This was regarded as the physiological type of bilirubin formed in the reticulo endothelial system from haemoglobin (see Fig 49). It was thought that haemobilirubin was picked out of the blood by the liver, and during its passage through the polygonal cells (*cf.* McNee) was changed in some way into cholebilirubin (see below). Hyperbilirubinæmia with a negative direct reaction is observed particularly in hæmolytic icterus, acholuric jaundice, etc., and in some cases of toxic jaundice without obstruction.

It was stipulated that sera giving a positive direct test contained some bilirubin which had passed through the liver cells but had then been reabsorbed owing to obstruction at some level in the biliary channels, this type of bilirubin was called "direct bilirubin" or "cholebilirubin". There might be gross anatomical obstruction as by a gall stone inside or by compression by a tumour outside the bile passage, or it might be necessary to postulate obstruction from inflammatory swelling or accumulation of mucus, etc. In many of these cases the direct reaction was not intense enough to account for the whole of the bilirubin in excess of normal, so there was both cholebilirubin and an excess of haemobilirubin, accordingly there was both obstruction and excessive breakdown of hæmoglobin to haemobilirubin or obstruction and accumulation of haemobilirubin in the blood owing to the inability of the damaged liver cells to pass it all through.

In the test tube when alcohol is used all the bilirubin is always converted into azobilirubin in Van den Bergh's reaction, so it was stated that alcohol converted non direct haemobilirubin into direct cholebilirubin.

There can now be little doubt that most of these theoretical conceptions were premature. Experimentally in dogs, Mann *et al* found that after removal of the liver first an increased indirect and then a direct reaction developed, showing that liver cells are not necessary for the formation of cholebilirubin. Clinically a positive direct reaction has been observed in some cases of acholuric jaundice,

in the absence of obstruction by pigment stones. Further in clinical work it is now generally agreed that a positive direct reaction is of little or no help in deciding whether obstruction exists. Hyperbilirubinaemia of several units with a negative direct test is usually associated with icterus of the hæmolytic type, but even that combination is not absolutely diagnostic. Hyperbilirubinaemias with delayed and increasingly positive direct reactions are certainly not limited to any one type of jaundice. There is really no generally accepted convention as to what exactly constitutes a positive direct test, as mentioned above, some normal sera develop a reddish tint after the direct addition of diazo reagent if they are observed for an hour. The writer's opinion is that the direct reaction is almost valueless in clinical work.

Chemically many investigations have been made on the two types of bilirubin (Andrewes, Collinson and Fowweather, Davies and Dodds, Newman, Roberts, Hunter, Griffiths and Kaye, Fowweather, Griffiths, among others), or more exactly on biological fluids or extracts thereof which contain bilirubin and do or do not give a direct reaction. The two types have never been isolated each in a state approaching purity, and unchanged. There may be two types, but it is equally possible that it is not the bilirubin but the impurities or other factors influencing the diazo reaction which differ, and that alcohol removes the interfering factors.

The writer is reminded of his experience with diazo tests for indican in urines in which the same diazo reagent is employed, though the acid mixture is finally made alkaline to obtain a characteristic pink or red colour (see p. 97 for reaction applied to serum). Some urines give a 'direct' test with the diazo solution many which do not may be made to do so by first precipitating with two volumes of alcohol and applying the test to the filtrate, in the language coined for Van den Bergh's test they give an indirect reaction for indican! Of course indican and bilirubin may or may not be analogous in behaviour.

Until more is known of the chemistry of bilirubin (cf. Lemberg) and of its diazo reaction in particular, it would be wise to set on one side the theories developed from the findings by Van den Bergh's test. In the meanwhile the quantitative indirect reaction is the most practicable method for estimating the (total) bilirubin of serum in clinical work. The direct test (qualitative or quantitative) is of doubtful value and in routine work might be omitted.

This suggestion fits in with the general experience of the value of Van den Bergh's test clinically. Its chief use is in patients having subicteric or peculiar yellow tints of the skin or conjunctiva, in that it enables a statement to be made as to whether or not there is an excess of bilirubin in the blood. So far as is known, of substances found in biological fluids, only bilirubin and biliverdin give the red colour with the diazo reagent in acid solution. In addition, the test gives a rough measure of the degree of hyperbilirubinaemia. This is often unnecessary in diagnosis, but may be useful in following progress.

It is stated (e.g., Hunter, Weiss) that the renal threshold is different for the two types of bilirubin, that for hæmobilirubin

Summary of Findings for Bilirubin and Urobilin in Blood, Urine and Faeces

Condition.	Blood		Urine		Faeces		
	Bilirubin n gm per 100 c.c. (Van den Bergh's Test)	Urobilin and -ogen by ordinary tests	Bilirubin	Urobilin and -ogen.	Bilirubin	Stercobilin and -ogen	Colour
Normal	0.1 to 0.5	None detectable by ordinary tests	0	None detectable by ordinary tests	0	+++	Brown
Excessive haemolysis (acholuric jaundice, pernicious anaemia, etc.)	Often exceeds 0.5	Trace	0 to trace	Trace to +++	0 or trace	++++ Increased	Dark brown
Gross diarrhoea	0.1 to 0.5	None detectable	0	None detectable	0 to trace	Normal or decreased	Liquid Pale brown
Partial obstruction to bile passages	Greater than 0.5	0	trace to +++	0 to trace	0	Decreased	Pale
Complete obstruction	Much greater than 0.5	0	+++	0	0	0 or mere traces	Very pale
Liver disease without obstruction	Often exceeds 0.5	0 to trace	0 to +	0 to trace	0	Often decreased.	Normal or pale
Liver disease with obstructive element	Exceeds 0.5	0	+ to +++	0 to +	0	Decreased	Pale



in hæmolytic icterus being higher—2.5 to 9 mgm per 100 c.c. (5 to 18 units)—than that for cholebilirubin in obstructive jaundice—1.5 to 2 mgm (3 to 4 units). In the writer's opinion this needs re-investigation, using the modern more sensitive tests (Hunter's, Fouchet's) for bilirubin in urine. From preliminary observations in his laboratory he is not convinced at present that there are two different thresholds, and certainly bilirubin can often be detected in the urine by the sensitive methods when the corresponding blood bilirubin is 0.5 to 1.0 mgm (1 to 2 units).

The main findings as regards bilirubin and its derivatives in blood, urine and feces are summarised in the table on p. 247.

### Bile-salts

Urine examination and the cycle of bile salts have already been discussed. Bile salts are found in the feces only in gross diarrhoea. Normal blood (Rowntree *et al.*) contains 2.5 to 6.0 mgm per 100 c.c. (as glycocholic acid). In obstruction the blood bile salts rise to about 15 mgm or less. With persistence of obstruction the percentage of bile salts in the blood falls, though it may remain slightly above normal. In other words, the blood shows changes parallel with those in the urine.

The method (Aldrich and Bledsoe) used in the above observations is an application of the Pettenkofer reaction to alcoholic extracts of blood. As the authors emphasise, it is not specific and is likely to give maximum values. It is still under trial, and its value in clinical work is not yet established, though it is obviously of the greatest importance in studying the metabolism of the bile salts. According to Rowntree bile salts are not the cause of the pruritus often observed clinically in jaundice.

Using Szilard's method much higher values for serum bile salts have been reported (Shattuck *et al.*, Katayama), the normal range being 5 to 12 mgm (as sodium glycocholate), and values as high as 200 mgm per 100 c.c. occurring in catarrhal jaundice. Using this method the renal threshold for bile salts appears to lie at about 20 mgm per 100 c.c. of plasma, thus accounting for the absence of bile salts from the urine when biliary obstruction is chronic.

When, owing to obstruction, bile salts do not reach the intestine, fat is not properly emulsified and absorbed, wherefore the blood does not contain the normal quantity of minute fat particles after a meal rich in fat. On this basis Brulé introduced his so-called *hæmoconia test*, in which the blood is examined under dark ground illumination. If the number of fat particles (hæmoconia) is greatly diminished after a suitable meal, it is deduced that bile salts are deficient or absent. The method is of value as a rough qualitative test.

### Cholesterol

Cholesterol is excreted virtually entirely in the bile (*cf.* Chapters V and XXIII). It is derived partly from the diet and partly endogenously, there being now little room for doubt that cholesterol

can be synthesised in the body, as well as being formed by the breakdown of old red blood corpuscles. Probably the bulk of the bile cholesterol is not reabsorbed in the intestine, but is reduced to coprosterol and excreted in the faeces. Estimation of coprosterol in the faeces, however, is a relatively complicated procedure, and so far as the writer is aware has not been attempted as a clinical test of obstruction to the bile passages.

Very contradictory conclusions have been published as to the clinical value of blood cholesterol determinations in diseases of the bile passages, but it is probably fair to state that significantly high values are encountered only in gross obstruction (*cf* Gardner and Gainsborough). Fox points out that the entire suppression of one day's bile output would only raise the blood cholesterol 10 mgm per 100 c.c., so that in the absence of obvious jaundice, and therefore of any serious grade of obstruction, it is unlikely that blood cholesterol estimations will be of any clinical assistance. When jaundice is present a high blood cholesterol points to the presence of obstruction, and thus may occasionally provide information of value, though usually the presence of obstruction will be diagnosed with certainty without resort to blood analysis. From the above it is easy to understand why blood cholesterol determinations have been so disappointing in cholelithiasis, which is the disease in which such analyses might have been expected to be of value, since gall stones so often contain very large proportions of cholesterol. For further discussions of blood cholesterol, see Chapters V and XIX.

### METABOLIC FUNCTION—(a) CARBOHYDRATES

One of the most important functions of the liver is its "glycogenic function," which term generally embraces both the formation of glycogen (glycogenesis) from monosaccharides (mainly glucose), and the breaking down of glycogen (glycogenolysis), into glucose. Obviously it is impossible to obtain any measure of the glycogen content of the liver in living man, and clinical observations are limited to blood and urine examinations for sugars. Further, such observations are limited to the peripheral blood, it being obviously impossible to secure portal blood.

Slight glycosuria is not uncommon in patients who have disease of the liver, but it is often difficult or impossible to decide whether damage to the liver or coincident pancreatic disease is the cause. The original "glucose tolerance test," which consisted of giving increasing doses of dextrose by mouth and noting the smallest amount which caused glycosuria, has fallen into disuse. The result depends upon the rate and degree of absorption from the intestine, the efficiency of the liver and of the pancreas, the kidney threshold, etc. A blood-sugar curve after 50 gm. of dextrose (see Chapter VII) the modern "glucose tolerance test," is likewise of little or no value as a test of liver efficiency, since other factors (*e.g.*, the pancreas) are often of more importance. A curve after 50 gm. of laevulose, however, is generally considered to be of more value, though here

again it is often impossible to assess the influence of coincident pancreatic disease. In completely dehepatised animals, extreme hypoglycaemia sets in at once, but nothing corresponding to this has been observed clinically in liver disease, although chronic hypoglycaemia has been reported in a few patients with extensive liver damage.

### The Lævulose (Fructose) Tolerance Test

The test is carried out in exactly the same way as is the glucose tolerance test (see Chapter VII), except that 50 gm of lævulose (fructose) are given instead of 50 gm of dextrose. The blood analyses are the essential part, urine tests for fructose being of

BLOOD SUGAR CURVES AFTER 50 gm OF LÆVULOSE  
IN HEALTH (BROKEN LINE) AND IN TOXIC JAUNDICE  
(SOLID LINE)

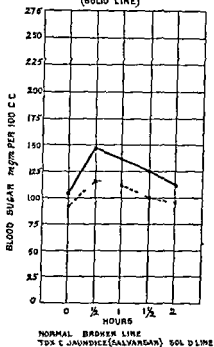


FIG 50

little or no importance, since a renal threshold for fructose is either low or non-existent. In fact, tests for reducing substances in the urine may be omitted in making the lævulose test though of course they should always be made as part of the routine examination of the patient.

Normally after 50 gm of lævulose the blood sugar does not rise significantly, probably due to the fact that the absorption of fructose from the intestine is slower than that of glucose. In other words, the blood sugar "curve" is a straight line or nearly so. It is now fairly generally agreed that the curve is not pathological, unless the difference between the highest and the initial blood sugar exceeds 30 mgm (*cf* Tallerman). If amounts greater than 50 gm are given the curve is higher and approaches nearer to the curve after a similar dose of glucose. The dose of lævulose therefore is important. Spence and Brett recommend 50 gm for a person of 80 kgm, 40 gm for 60, and 30 gm for 40 kgm. It is a common practice, however, to use 50 gm for all adults, and this appears to be satisfactory. In children the writer has found about 1 gm of lævulose per kgm suitable.

In certain cases of hepatic disease the curve is raised and prolonged (Fig 50), but in quite a number of patients with extensive involvement of the liver it is not. It has been useful in toxic liver damage (*e.g.*, salvarsan, chloroform, phosphorus, atophan poisoning) and is often positive in catarrhal jaundice and may be in obstructive jaundice, in both of which it is generally superfluous in making a

diagnosis. Indeed, in the extensive investigations of Greene, Rowntree, Snell, Walters, *et al*, the general conclusion is reached that a positive finding (*i.e.*, a raised and prolonged curve) is occasionally of value in combination with other tests of hepatic efficiency, but that a negative result is of little or no significance. Several workers have found the laevulose tolerance test useful in assessing the risk of poisoning when treating syphilitic patients with salvarsan or its derivatives, and occasionally in following the progress of a case of toxic jaundice (*e.g.*, salvarsan, lead or atophan poisoning). The writer would now reserve the test for this type of problem and for special investigations, since it is time consuming and of doubtful value in routine work.

The blood sugar curve after laevulose is invariably raised and prolonged in diabetes mellitus, presumably due to a deficiency of endogenous insulin. It is possible that the positive results in toxic jaundice, etc., are likewise due to pancreatic damage. It has been assumed that in health the liver changes fructose into glycogen so rapidly that the sugar in the peripheral blood does not rise, and that in liver disease the hyperglycaemia is due to a reduction of the number of liver cells, or due to a depression of their function. But it has been shown that fructose is less rapidly absorbed from the intestine than glucose, and this alone may account for the "straight line curve" in health. In those patients with liver disease and hyperglycaemia after fructose, the hyperglycaemia may be due to coincident pancreatic lesions and endogenous insulin deficiency. It is possibly significant that many cases with very gross liver damage but without jaundice give a negative laevulose test, and that the greatest proportion of positive results is obtained in toxic jaundice, in which it is not unlikely that there is a coincident pancreatic lesion. Mann *et al*, from their experimental work, conclude that fructose tests cannot be regarded as specific for hepatic function.

The above remarks apply to the results of determining the total sugar in the blood after the administration of fructose. Recently Roe and Scott have published methods for the estimation of the fructose itself in the blood.

### The Galactose Tolerance Test

Galactose has been used to test hepatic efficiency for many years, particularly in Germany, but has never been universally adopted. The subject has recently been reviewed by Shay *et al*, whose work has again roused considerable interest in this sugar. In spite of some evidence to the contrary, the following is a fair summary of the points in its physiology with regard to liver function testing. It is rapidly absorbed from the intestine, even more readily than glucose, the ratio of the rates of absorption of galactose, glucose and fructose being respectively 110, 100 and 43 (Corn). It is not, however, so easily converted into glycogen by the liver as the other two monosaccharides, and if given in excess of the liver's immediate capacity, it passes into the general circulation, where again it is less readily utilised (by the muscles, etc.) than the other

two, and is therefore excreted in the urine. About 40 gm of galactose is the maximum dose with which a liver whose cells are not damaged can deal, a dose which seems to be independent of sex, age and body weight. After this amount in health the peripheral capillary blood sugar usually rises just as much as, and even higher than after the same dose of glucose, but this rise is presumably due mainly to glucose formed from liver glycogen, and not to galactose, because not more than 3 gm appear in the urine during five hours after oral administration of 40 gm, in spite of the fact that there is no renal threshold for galactose. In the case of this sugar, then, it is the amount excreted in the urine which matters, the blood sugar level and blood sugar curves are of no assistance.

Clinically the value of the test in the form proposed is that it is of some help in differentiating jaundice due to damage to liver cells from that due to mechanical obstruction or hæmolytic in origin. Thus Shay *et al* quote from Wörner 133 positive results in 165 cases of catarrhal jaundice, but only 9 positive out of 132 cases of jaundice due to tumours and gall stones. Naturally the differentiation is not absolute, because the liver cell damage may not be severe enough in catarrhal jaundice at the time of the test to give a positive result, and in long continued obstruction the secondary damage to the liver cells will produce a positive finding. As usual the laboratory report can only be interpreted in the light of the clinical evidence, and in some instances interpretation must remain difficult or a matter of personal opinion. Clearly in diabetes mellitus or in any other pre-existing glycosuria, it is of no use merely to estimate the urinary output of total reducing substances after a dose of 40 gm of galactose, but Shay *et al* maintain that the test is still of value in such cases if the glucose is first removed from the urine by fermentation.

The technique recommended by these authors is as follows. No food is allowed overnight or at breakfast time. In the morning the subject empties the bladder completely, and then drinks 40 gm of pure galactose dissolved in about 500 c c of water with lemon to flavour. Subsequently urine is collected hourly for a total of five hours though the intervals need not be exactly of sixty minutes so long as the five hours collection is complete. The initial sample is tested to make sure of the absence of pre-existing glycosuria. Each of the subsequent specimens is tested qualitatively for reducing substance; the positive samples are mixed, the total volume is measured, the sugar is determined quantitatively by Benedict's method (25 c c of Benedict's quantitative reagent are reduced by 0.054<sup>1</sup> gm of galactose), and the five hours' output of sugar is calculated. Normally 0 to 3 gm are excreted.

In patients with glycosuria, a sample of the combined and mixed urine is first treated with yeast to remove the glucose. One part of urine is mixed with 7.7 parts of the yeast suspension, kept for forty five minutes at room temperature, cleared by centrifuging or

<sup>1</sup> 0.054 is the equivalent quoted by Bensley (*Canal Med Assoc J.*, 1933, 33, 360). G. D. Seombe in the writer's laboratory makes it 0.058.

filtering, and the galactose content determined by Benedict's method. To prepare the 10 per cent yeast suspension, a weighed amount of fresh commercial yeast is suspended in about 5 parts of water and centrifuged. The deposit is resuspended in a similar volume of water, and the process is repeated until the supernatant fluid is clear and colourless and does not reduce Benedict's qualitative reagent. Finally the deposit is suspended in water, of which 10 c.c. is used for every 1 gm. of yeast originally weighed out. Of the 77 parts of suspension, 0.7 is allowed for the volume of the yeast cells, the urine dilution is therefore 1 in 8.

Alternatively the galactose may be estimated polarimetrically (specific rotation  $[\alpha]_D^{20} + 80.5^\circ$ )

### METABOLIC FUNCTION—(b) PROTEINS

The discovery of leucine and tyrosine in the urine in acute yellow atrophy is a very old observation, but it has been shown that the necrosis is responsible for these. They occur when there is gross destruction of any tissue, and not only of the liver (Cf Chapter IV).

Protein is hydrolysed to amino acids in the intestine. The amino acids are absorbed into the portal blood. Some are utilised by the tissues. Some are deaminised by the liver, the ammonia being converted into urea. Several attempts to devise means of measuring the functional efficiency of the liver on the basis of the above facts have been made. Thus the nitrogen partition of the urine or of the blood, the amino acid nitrogen of the blood or the output of amino acids in the urine, the urea percentage of the blood or the output of urea, blood urea curves after a meal rich in protein or after the ingestion of amino acids (glycine (Witts)), and excretion of amino acids after their ingestion (glycine, alanine, asparagine), have all been investigated, and have all been of little or no value clinically. This is readily understood from the experimental work of Mann *et al*. In the liverless dog there is an increase in the blood of a substance giving the colour reactions of uric acid, but in hepatic disease in man the blood uric acid is not raised except when there is gross destruction of tissue (acute yellow atrophy).

It may be concluded therefore that there is no satisfactory clinical test based on the share of the liver in protein metabolism.

### METABOLIC FUNCTION—(c) FATS

The liver plays an important part in fat metabolism in view of the excretion of bile salts which are necessary for the proper emulsification and absorption of fats. It also stores and dehydrates fats, which are mobilised from the fat depots (subcutaneous tissues, etc.), but no clinical test of this function has been devised.

The importance of the bile salts has already been mentioned in reference to the urine, the blood, and the cycle of bile salts. Their influence on the differential estimation of fat in faeces remains for discussion.

### Fat in Fæces

When bile salts are prevented from entering the intestine, the absorption of fats is defective, but the splitting by pancreatic lipase is usually unaltered. In obstructive jaundice therefore the total fat is excessive, but the proportion of split fat is normal unless the obstruction also includes the pancreatic duct.

Normally, of the dried fæces not more than one fourth is fat, and of that fæcal fat not more than one fourth is unsplit neutral fat. (For proportions in children's fæces, see Chapter XXIV.) In obstructive jaundice the total fat often rises to 50 per cent or higher, but in uncomplicated obstruction the split fat (free fatty acids plus soaps) generally constitutes 70 per cent or more of the total fat. If there is also obstruction to the pancreatic duct or gross destruction of the pancreas, the split fat is less than 70 per cent of the total fat, sometimes much less, e.g., 50 to 20 per cent.

Whilst the above may be taken as a rough guide, the interpretation of fæcal fat analyses in lesions of the liver and biliary passages is often difficult. This is particularly the case when coincident pancreatic lesions are possible or probable. The influence of the amount of fat in the diet, and the possible lipolytic action of bacteria have also to be considered. For further discussion and the methods of estimation, see Chapters XXIV and XIII.

In obstructive jaundice the fæces are pale, partly owing to the failure of bilirubin to enter the intestine and partly owing to the excess of fat. The stools are often bulky, due mainly to the large proportion of fat. The passage of bulky fatty looking stools is commonly called "steatorrhœa," though strictly the term should be limited to the passage of fatty fæces which are liquid when passed and set on cooling (see Chapter XIII).

In hæmolytic jaundice the stools are dark owing to the excess of stercobilin and a differential estimation of fat gives results within normal limits.

### Blood Lipase

The liver, the pancreas, and all the tissues in which fat is stored contain lipases. The blood plasma also contains traces of lipases. Whipple *et al* found that the plasma lipase in dogs rose considerably after liver poisoning with chloroform, phosphorus or hydrazine. In man an increase in plasma lipase has been reported in the more acute types of liver disease (eclampsia, salvarsan poisoning, acute yellow atrophy, catarrhal jaundice, etc.) but is uncommon in chronic hepatic lesions.

The methods of estimation employed in clinical work are unsatisfactory, and the physiological basis of the test as a measure of hepatic disease has not been established. The influence of pancreatic lesions and of other possible factors has not been determined. Further investigation is required.

## • THE ANTITOXIC POWER OF THE LIVER

Unabsorbed food and the digestive fluids poured into the gut are subject to putrefaction by the enormous quantities of bacteria, particularly in the large intestine. Certain toxic products are formed by the bacteria, and are absorbed, and would cause damage if they were not rendered inert by the liver or possibly by other tissues. In addition, certain drugs and poisons are similarly made harmless. The chief methods of detoxication are oxidation, reduction, and combination (conjugation) with other substances to yield non toxic compounds. Some toxic products may be excreted in the bile. It is with reference to conjugation that most attempts to devise tests of liver efficiency have been made. The two principal methods of conjugation are with sulphates and with glucuronates. Thus indoxyl, which is formed by the action of bacteria on tryptophan, may be conjugated with either, yielding potassium indoxyl sulphate (indican) or salts of indoxyl glucuronic acid. The degree of indicanuria, however is useless as a liver function test. It depends also on the amount of tryptophan exposed to the action of bacteria and the type of bacterial flora. Indicanuria is sometimes practically absent, sometimes very marked in liver disease. Measurements of indican excretion after known doses of indole, or of the increase in excretion of ethereal sulphates after the administration of thymol, have also failed as clinical tests of hepatic efficiency. The same general conclusion applies to other substances which have been tried in order to provoke conjugation e.g., camphor, cresol, guaiacol, menthol, paracresol, phenol, salicylic acid. The explanation of this may be that conjugation can occur in other tissues than the liver. Mann *et al* found in the liverless dog that there was no diminution of the power to conjugate phenol. Though obviously, from its anatomical position, the liver in health must be a very important organ for detoxication it is quite likely that in disease other tissues can take on the function, for which reason tests of liver efficiency depending on conjugation have been disappointing.

Widal *et al* introduced another test which, in their opinion, depended on the power of the liver to deal with proteoses or peptones derived from the breakdown of proteins in the intestine. If the liver was diseased they suggested that these protein products would pass into the general circulation, and cause signs of protein shock or a "hæmoclastic crisis" (leucopenia, fall of blood pressure, etc.). There is, however, no evidence that proteoses or peptones can pass into the portal circulation, and the general conclusion from both experimental (Mann) and clinical observations is that the test is of no value as a measure of hepatic efficiency.

## HÆMOPOIETIC FUNCTIONS

The frequency of hæmorrhages in hepatic disease and the grave risk of post-operative oozing of blood in jaundiced patients is an



old clinical observation. In such cases the coagulation time is generally normal, but the bleeding time is often increased, and its determination provides a useful warning before operation. Intravenous injection of calcium chloride is a valuable prophylactic measure. Hæmorrhage is especially troublesome in obstructive jaundice. Several explanations of these observations have been attempted. Though the total calcium of the serum is practically always normal, it has been suggested that less ionised calcium is available for coagulation owing to its partial fixation by the excess of bilirubin in circulation. That this is an unsatisfactory explanation, however, is readily shown by calculating the amount of calcium which could possibly be fixed by the circulating bilirubin.

Foster and Whipple have concluded that the liver is the main if not the only source of fibrinogen, but they have found that tissue injury (including inflammation) is an efficient stimulus to increased fibrin production. It is clear therefore that a determination of blood fibrinogen cannot be used as a measure of hepatic efficiency. At the same time a deficiency of fibrinogen due to liver disease may be a cause of the delay in coagulation.

It has been found that when the blood fibrinogen is low the coagulation time may be normal, but the clots may be too delicate to prevent hæmorrhage, and Mann *et al* have noted much oozing several hours after complete excision of the liver in dogs.

## EXCRETION OF FOREIGN SUBSTANCES

Bile may be regarded as the excretion of the liver in the same way as urine is the excretion of the kidneys. The liver is the main excretory path in the portal circulation, the kidneys in the general circulation. The excretion of the normal ingredients of bile has already been discussed. In addition the liver may throw out, in the bile, organisms (*e g*, typhoid), drugs and poisons. The bile would appear to be the chief medium for the removal of fats, lipoids, and fat soluble dyes (*e g*, Sudan III). Lastly, certain water-soluble dyes are excreted mainly or entirely by the liver, just as others are by the kidneys (*cf* Chapter V), and this observation is the basis of a useful method of measuring liver efficiency.

Either the fæces or the bile obtained by duodenal intubation may be examined for the dye, which is administered parenterally, but the most satisfactory procedure has been a determination of the amount of dye left in circulation at given intervals after intravenous injection. The dyes employed include phenoltetra-chlorophthalein, bromsulphalein, and rose bengal. Graham has pointed out that one injection of phenoltetraiodophthalein may be used both for the blood dye test and for cholecystography. Just as, broadly speaking, there is a parallelism between the excretion of urea and phenol red in the urine, so there is between the excretion of bilirubin and of dyes in the bile. Perhaps, partly for this reason and partly for the natural hesitation in employing an injection which, if badly made, may cause severe local reactions and even

sloughing, the dye tests of liver function have not so far been extensively employed in this country. The latter objection, however, does not apply to bromsulphalein, which is non toxic and non-irritant.

#### Phenoltetrachlorophthalein (Rosenthal)

The patient is weighed and 5 mgm of the dye per kgm are injected intravenously. Blood is taken from the opposite arm fifteen minutes, one and two hours later. Serum or plasma is separated in the usual way, hæmolysis being avoided. If, however, hæmolysis does occur the hæmoglobin may be precipitated with acetone, though the extraction of the dye by acetone is not quite complete (Bloom and Rosenau). A trace of alkali is added to each of the three sera to develop the maximum colour. Determinations are made colorimetrically by comparison with standard tubes of the dye, the technique being similar to that described for bromsulphalein in the next section. Results are expressed as percentages of an assumed initial concentration of the dye in the plasma. Variations in plasma volume introduce slight inaccuracies, which may generally be disregarded.

Normally, only 2 to 7 per cent of the dye is left in circulation fifteen minutes after injection and less than 3 per cent at the end of an hour. The hourly period is the one usually selected. In liver disease, after this interval as much as 50 per cent of the dye may still be in circulation, and figures over 20 per cent are common.

Normally, only the merest traces appear in the urine, and inconstantly. In some cases of liver disease larger quantities are excreted in the urine, owing to dye retention in the blood.

#### Bromsulphalein<sup>1</sup> (Rosenthal and White, Bulmer)

Bromsulphalein, or phenoltetrabromphthalein sodium sulphonate, is a whitish powder which is freely soluble in water, non irritant and non toxic in ordinary doses. After intravenous injection nearly all is excreted in the bile, mere traces appearing in the urine.

The patient is weighed and 2 mgm of the dye per kgm are injected into a vein of one arm. A 5 per cent solution is usually employed and is injected slowly (one or two minutes) with a 5 c c syringe. At the end of thirty minutes about 5 c c of blood are withdrawn from the other arm. The serum is separated and placed in two small test tubes of the same bore as the standard tubes. To one of the tubes 1 or 2 drops of 5 per cent sodium hydroxide are added to bring out the colour of the dye, and to the other the same volume of distilled water, unless there is hæmolysis, in which case 1 or 2 drops of 10 per cent hydrochloric acid are added, thereby changing the hæmoglobin to acid hæmatin, which is of approximately the same tint as that of the alkaline hæmatin formed in the alkalinised serum. The colour of the unknown is then compared with that of the standard in a comparator, a tube of distilled water being placed

<sup>1</sup> A sterile 5 per cent solution of the dye in ampoules is prepared by Hynson, Westcott and Dunning.

behind the unknown, and the tube of serum plus distilled water (or HCl) being placed behind the standard tube. In this way compensation is effected for colours in the unknown other than that due to the dye. Slight hæmolysis is permissible owing to this method of compensation, but if gross hæmolysis has occurred during collection of the blood, direct colour comparison is difficult, and the dye must be extracted with acetone, or the test must be repeated.

The 100 per cent standard is prepared by dissolving 4 mgm of the dye in about 70 c c of distilled water, adding 5 c c of  $\frac{1}{2}$  per cent sodium hydroxide (or its equivalent), and making up to 100 c c with distilled water. A series of standards from 5 per cent upwards is prepared by dilution with 0.025 per cent sodium hydroxide, and stored in the dark.

The 100 per cent standard represents the amount of dye that would be present in the blood plasma immediately after the injection. It is an arbitrary standard calculated on an assumed blood volume as follows —

Let the body weight be  $x$  kgm. Then the quantity of dye injected is  $2x$  mgm. It is assumed that the plasma volume is 50 c c per kgm of body weight (*cf* Chapter XIX). Therefore the total plasma volume is  $50x$  c c.

Since  $50x$  c c of plasma contain  $2x$  mgm of dye,

$$1 \text{ c c of plasma contains } \frac{2x}{50x} \text{ mgm of dye,}$$

$$100 \text{ c c of plasma contain } \frac{2x \times 100}{50x} = 4 \text{ mgm of dye,}$$

immediately after injection. Therefore the 100 per cent standard is made up with 4 mgm of dye per 100 c c of solution.

Normally, half an hour after injection, the amount of dye left in the plasma is *nil* or a mere trace. In diseases other than of the liver, dye retention does not exceed 5 per cent. In hepatic lesions all grades of retention are encountered, even up to 100 per cent.

## SUMMARY OF TESTS OF CLINICAL VALUE

It is convenient to summarise the tests under the heading of the different body fluids or excretions which are examined.

**Urine** *Routine chemical examination* with especial reference to bilirubin, urobilinogen, urobilin, bile salts and glucose. Examination of deposit for leucine and tyrosine in suspected acute yellow atrophy.

**Blood** (1) *Bilirubin*, using Van den Bergh's test, Fouchet's reaction, or the icterus index. The first is of the greatest value in most instances. One of the three should always be performed on patients with subicteric tints or peculiar yellow colours, and in jaundice. Even when clinically there is no jaundice, it is advisable to carry out Van den Bergh's reaction as a routine in all patients with possible hepatic disorders. Latent jaundice may be detected as a result.

(ii) *Dye tests* (bromsulphalein or phenoltetrachlorophthalein) do

not appear to have been employed much in Great Britain, but in American literature they seem to rank second to Van den Bergh's reaction

(iii) The *laevulose tolerance test* may or may not be a test of liver efficiency, but has been valuable in assessing the risks of poisoning by salvarsan and its derivatives, and in following the progress of toxic jaundice

(iv) The *urinary galactose tolerance test* promises assistance in differentiating toxic and infective jaundice from other types

(v) Determination of the *coagulation time* and the *bleeding time* in jaundiced patients before operation

**Examination of the duodenal contents** (Chapter XXIII), is of limited value in cholelithiasis and cholecystitis, and occasionally in other disorders

**Fæces** Tests for *stercobilinogen plus stercobilin* in obstructive jaundice as an indication of the degree of obstruction *Differential estimation of fat* is occasionally of value, particularly when a coincident pancreatic lesion is suspected

## CONCLUSION

From the clinical point of view the current tests of liver efficiency are disappointing. In a small proportion of cases only do they add information of value to the clinical examination. Since bilirubin is a coloured compound, estimations of blood bilirubin are not nearly so essential in liver diseases as are estimations of blood urea in kidney diseases. The writer's routine practice is to examine thoroughly the urine and to perform Van den Bergh's test. He reserves all other methods for special problems, as indicated above, or for particular investigations, since it is the duty of those who have opportunities in hospitals to work systematically to assess the value of tests and to gain new knowledge.

## TECHNICAL

### Icterus Index, Bilirubin Index, Plasma Colour, or Meulengracht's Test.

**Principle** The colour of the plasma or serum is compared with a standard solution of 1 in 10,000 potassium dichromate, the colour intensity of which is taken as unity (Index = 1)

#### Standard Solution.

Potassium dichromate	.	0.1 gm
Concentrated sulphuric acid		0.1 c c
Distilled water	. to	1,000 c c

The sulphuric acid is added as a preservative, in this dilution it does not influence the tint

If preferred a series of solutions which are multiples of the above may be prepared so as to obtain standard tubes corresponding to indices of 1 up to 10, or 1 up to 20, and so on. Thus a stock solution

of potassium dichromate 0.1 gm, concentrated sulphuric acid 0.1 c.c., water to 100 c.c. is made up. From this the "9" standard is prepared by adding 1 volume of water to 9 volumes of the stock solution, the "8" standard by adding 2 volumes of water to 8 volumes of stock solution, and so on.

**Technique.** Serum or plasma is obtained in the usual way. Three things are to be avoided, *hæmolysis*, *lipæmia* and *carotinæmia*. The following are important if hæmolysis is to be prevented. All apparatus (needles, syringe and tubes) must be dry (liquid paraffin is useful to lubricate the syringe). Excessive venous constriction must not be applied. The blood must neither be forcibly squirted through the needle, nor centrifuged at rapid rates, lest hæmolysis result from mechanical injury to the corpuscles. Excess of anti-coagulants must not be employed (see Chapter XVII on collection of blood), but even with the greatest care many plasmas will show the absorption bands of oxyhæmoglobin if examined spectroscopically, it is easier perhaps to secure serum without hæmolysis. The pale reddish tinge makes accurate colour comparisons impossible. This is the main difficulty in an otherwise extremely simple test. Hæmoglobin may be removed, together with other proteins, by acetone (see below). If the blood is obtained in the morning after a night's fast lipæmia and carotinæmia are generally absent. In some pathological states, however, lipæmia persists throughout the twenty-four hours under any condition that may be imposed. In extreme cases of carotinæmia (which are rare) it may be necessary to order a vegetable free diet for two or three days beforehand. Even this sometimes fails. On very rare occasions other colouring matters are present in the serum.

The colour of the plasma is compared with the colour of the standard by placing unknown and standard solution in tubes of identical bore (e.g. "cordite" tubes). If the unknown is more yellow than the standard 1 c.c. is taken and diluted with physiological saline solution adding  $\frac{1}{2}$  or 1 c.c. at a time, until the colour matches that of the standard. Thus, suppose 5 c.c. of saline have to be added. Then the plasma has been diluted 1 in 6. Therefore, the colour of the original "unknown" was six times that of the standard. Therefore the *icterus index* is 6. Instead of diluting the unknown till it matches the standard, the two may be compared in a colorimeter in the usual way. If, however, the colour of the unknown is much more intense than that of the standard, it may be impossible to compare the two in this way. As in all colorimetric work, the colours of the unknown and standard solution should be approximately the same if accurate results are to be obtained.

Alternatively the plasma may be compared with the series of standards, the most suitable standard being selected for the final comparison. In practice the first method is to be preferred. The tint of the stronger standards often does not match that of the unknown, and yet after suitable dilution there may be no difficulty in comparing the unknown with the 1 in 10,000 dichromate standard.

A glass disc of suitable colour may be used as standard (Preston) The disc should be checked against the dichromate solution

**Use of Acetone to Remove Hæmoglobin** Precipitate the proteins in 1 volume of plasma or serum by the addition of 1 to 2 volumes of acetone Filter or separate the proteins by centrifuging Compare the colour of the filtrate or supernatant fluid with that of the standard as described above For dilution use a mixture of 1 part of water with 2 parts of acetone Allow for the dilutions when calculating the result Thus, 2 c c of plasma were treated with 4 c c of acetone, 2 c c of filtrate required the addition of 2 c c of 67 per cent acetone to match the 1 in 10,000 dichromate standard Therefore, the total dilution was

$$\frac{2}{6} \times \frac{2}{4} \text{ or } \frac{1}{6}$$

Therefore, the icterus index of the original plasma was 6 A little bilirubin tends to be adsorbed on the protein precipitate In many cases, therefore, the result obtained after acetone extraction is slightly low

Ernst and Förster have determined the relation between the concentration of the dichromate solution (and, therefore, the icterus index) and the bilirubin content in mgm per 100 c c

Pot Dichromate	Icterus Index	Bilirubin mgm per 100 c c
1 in 6 000	1 67	0 329
1 in 6,667	1 50	0 299
1 in 7,500	1 33	0 269
1 in 8,571	1 16	0 240
1 in 10,000	1 00	0 210
1 in 12,000	0 833	0 180
1 in 15 000	0 667	0 149
1 in 20,000	0 500	0 117
1 in 30,000	0 333	0 090

Except in the case of the very low values, the icterus index is approximately five times the number of milligrammes per 100 c c

#### Fouchet's Test

**Principle** The proteins in serum or plasma are precipitated by trichloroacetic acid The bilirubin is adsorbed on, and carried down with, the protein precipitate The adsorbed bilirubin is oxidised by ferric chloride The precipitate therefore turns green, greenish-blue or blue (biliverdin or cholecyanin)

#### Technique

Fouchet's reagent { Trichloroacetic acid . 25 gm  
Distilled water 100 c c  
10 per cent ferric chloride 10 c c

To 1 volume of serum or plasma add 1 volume of Fouchet's reagent The mixture may be made in a small evaporating basin

or on a porcelain plate. Shake thoroughly. A white precipitate is formed instantly, and this turns green slowly if little bilirubin is present. If there is much bilirubin the precipitate rapidly turns green or blue. If the precipitate remains white for twenty minutes or longer the reaction is negative. The test may be performed with as little as 1 drop of serum. The blood may therefore be obtained by puncture of the ear or finger. Three drops of serum and 3 drops of reagent are usually employed.

### Van den Bergh's Test

The technical details have been modified so many times, and the test is done in such a variety of ways in different laboratories, that the writer cannot hope to cater for all. The following simplified account describes the technique in routine use in his laboratory, and its choice is supported by the work of Godfried whose paper should be consulted for references to other methods.

### Principle

**DIRECT REACTION** Serum or plasma is treated with freshly-prepared diazo solution *p* sulpho phenyl diazonium chloride in acid solution. Azobilirubin is formed, and in positive reactions the treated plasma is red or reddish violet.

**INDIRECT REACTION** Serum or plasma is treated with the diazo solution and then with alcohol and saturated ammonium sulphate. The proteins are thus precipitated and the concentration of azobilirubin in the supernatant fluid is determined colorimetrically by comparison with the colour of a standard solution of cobaltous sulphate.

### Solutions

#### *Diazo reagent*

(A)	{ Sulphanilic acid	1 gm
	{ Concentrated hydrochloric acid	15 c c
	{ Distilled water	to 1 000 „
(B)	{ Sodium nitrite	0.5 gm
	{ Distilled water	to 100 c c

For use mix 25 c c of A with 0.75 c c of B (or 10 c c of A with 0.3 c c of B). This mixture of A and B is the diazo reagent, and must be made on the day of the test.

#### *Cobalt standard*

Dissolve 2.161 gm of anhydrous cobaltous sulphate in distilled water to 100 c c.

The colour of this solution corresponds to that of 1 in 200 000 bilirubin or 1/2 mgm of bilirubin per 100 c c, or 1 Van den Bergh unit. It lasts for a few months if stored in the dark.

**Collection of Blood** Serum or plasma is obtained in the usual way, the greatest care being taken to avoid hæmolysis, it must be free from hæmoglobin as judged by the unaided eye otherwise the following method will be unsatisfactory (see also under "Remarks")

**Direct Test** To 1 c c of serum or plasma in a centrifuge tube add 0.5 c c of diazo reagent, and note the time (start a stopwatch) Observe the change of colour, if any, and the time taken for its development Report what is observed, *e.g.*, "mixture became red in thirty seconds, and bluish violet in two minutes" If no colour change occurs within half an hour the reaction may be termed negative It is a good plan as a routine, and certainly in all difficult cases to put up a comparison tube simultaneously containing 1 c c of serum and 0.5 c c of water

**Indirect Qualitative Test.** This is of little use, since the great majority of sera after treatment with alcohol give some colour with the diazo reagent, and a negative indirect test is of little or no clinical value

**Indirect Quantitative Test** (See McNee and Keefer) To the mixture of 1 c c of serum and 0.5 c c of diazo reagent already made for the direct test

add

2.5 c c of alcohol (absolute or 96 per cent)

1.0 c c of saturated ammonium sulphate

**Mix** Stand for a few minutes to allow the precipitate to flocculate **Centrifuge** Separate the supernatant fluid of which take a known volume (1 or 2 c c) and compare with the cobalt standards which are prepared by dilution of the 2.161 per cent anhydrous cobaltous sulphate Standards and unknown are of course placed in tubes of identical bore The standards in use in the writer's laboratory are 1.0, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2 and 0.1 units If the supernatant fluid is of a deeper colour than the 1.0 unit cobalt standard, dilute it with 67 per cent alcohol (alcohol 2, water 1 part) until it matches and note the volume required

The dilution of the serum in the preparation of the supernatant fluid is 1 in 4, and not 1 in 5, because the saturated ammonium sulphate remains as a separate layer (1 c c) and does not contain azobilirubin

The following is an example of the calculation —

2 c c of supernatant fluid were taken, to which had to be added 1.5 c c of 67 per cent alcohol to match the 1 unit standard

First dilution of serum (preparation of azobilirubin) is 1 in 4

Second dilution (matching of colour) is 2 in 3.5

$$\therefore \text{total dilution is } \frac{1}{4} \times \frac{2}{3.5} = \frac{1}{7}$$

The colour of 1 in 7 treated serum matches the 1 unit standard, therefore the original serum must have contained 7 units or 3.5 mgm per 100 c c

**Remarks** An attempt may be made to perform the direct test quantitatively by comparing the colour of the 1 c c of serum plus 0.5 c c of diazo reagent with the cobalt standards, dilution when required is made with distilled water, and tubes of identical bore are employed Thus suppose 6.5 c c of water have to be added to match



the 1 unit cobalt standard the total dilution is 1 in 8 wherefore the original serum contained 8 units or 4 mgm of bilirubin per 100 c c. At times the tints of unknown and standard are so different that colorimetric estimation is impossible.

Visible hæmolysis interferes with both direct and indirect reactions. The comparison tube (1 c c serum and 0.5 c c water) makes a rough opinion possible in the direct test if the hæmolysis is slight but gross amounts of hæmoglobin spoil it altogether. In the technique recommended for the indirect test hæmoglobin is not precipitated but converted into acid hæmatin the brown or yellow tint of which spoils the colorimetric estimation. In the older technique in which 1 c c of serum was first treated with 2 c c of alcohol hæmoglobin was precipitated so that it did not interfere with the colour on subsequent treatment with the diazo reagent, this older technique is not recommended however because very serious errors may arise from loss of bilirubin by adsorption on the protein precipitate (*cf* Godfried) it is much better to obtain another sample of serum without hæmolysis.

Messrs The Tintometer Ltd have made a disc containing nine coloured glasses which match the cobalt standards so that the indirect quantitative estimation of bilirubin can be made in the Lovibond comparator which is illustrated on p. 296. These glasses are permanent in hue intensity and brightness and will ensure uniformity in the laboratories adopting them. They correspond to 0.2, 0.4, 0.6, 0.8, 1.0, 1.25, 1.5, 1.75 and 2.0 mgm of bilirubin per 100 c c of serum. They have proved of great value in the writer's laboratory where they are in daily use. It is no longer necessary to prepare cobalt standards at intervals the glass colour standards are permanent and the comparator is useful also for other methods.

The technique for the comparator is the same as that described above. Transfer the supernatant fluid to the right hand tube in the Lovibond comparator and compare its colour with that of the coloured glasses in the disc.

If the bilirubin is less than 2 mgm per 100 c c rotate the disc till the correct colour match is obtained and read off the result. It is easy to judge colours about midway between two consecutive ve coloured glasses. It is unnecessary to put a tube of distilled water behind the coloured glass the effect in any case is very slight.

If the bilirubin exceeds 2 mgm per 100 c c transfer 1 c c of the supernatant fluid to another of the comparator tubes and dilute it with 1 or 2 or 3 etc. c c of 67 per cent alcohol until it matches one of the coloured glasses. Multiply the glass reading by the dilution. Thus 1 c c of supernatant fluid required 4 c c of 67 per cent alcohol to match the 1.5 mgm glass therefore dilution was 5 fold and bilirubin was  $5 \times 1.5 = 7.5$  mgm per 100 c c.

The glass labelled 2 mgm matches a 2.161 per cent solution of anhydrous cobaltous sulphate placed in a comparator tube.

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## CHAPTER XIII

### TESTS OF PANCREATIC EFFICIENCY

**Books and Special Articles.** Coope's *The Diagnosis of Pancreatic Disease*

Weiss's *Diseases of the Liver, Gall Bladder, Ducts and Pancreas*

Barton's *Manual of Vital Function Testing Methods and Their Interpretation*

*Critical Review The Diagnosis of Pancreatic Disease* Sladden, A F S, *Quart J Med*, 1914, 7, 455

*The Diagnosis of Disease of the Pancreas* Garrod, A E, *Brit Med J*, 1920 1, 459

*The Diagnosis of Diseases of the Pancreas, with Special Reference to Diastase in the Urine* Wallis, R L M, *Quart J Med*, 1920, 14, 57.

SEVERAL references were made in the previous chapter to the close connection between the liver and bile passages on the one hand, and the pancreas and its ducts on the other hand. It is natural therefore to consider efficiency tests of the pancreas immediately following efficiency tests of the liver. Diseases of the whole pancreas, however, are much less common than diseases of the liver or of the kidneys. The same general principles apply here as outlined for the other two organs in Chapters V and XII. The tests yield abnormal results only when the disease of the organ is extensive. The gland possesses a large "reserve power" and in interpreting the results of tests the possible influence of non-pancreatic factors must be considered, and in particular the likelihood of coincident disease of the liver or bile passages.

Tests of pancreatic efficiency are indicated as an extension of the clinical examination of patients with suspected disease of the organ, or in an attempt to obtain some measure of the degree of damage in known pancreatic disease. Examination of the urine seldom leads to requests for pancreatic efficiency tests, for, though glycosuria at once suggests diabetes mellitus and therefore a lesion of the pancreatic islets, glycosuria is an uncommon sign of disease involving the whole organ.

#### PANCREATIC EFFICIENCY TESTS IN DIABETES MELLITUS.

In the majority of diabetics the islets of Langerhans alone are seriously affected. In some, judging from post mortem histological examination, there is no evidence of damage to the islets, in which case either a functional inefficiency of the islets or a disturbance of the balance between the different ductless glands, or a disorder of other mechanisms controlling the output or availability of the internal secretion of the pancreas must be assumed.

In most cases of diabetes efficiency tests reveal no abnormality of the external secretion of the pancreas, and it is assumed that the lesion or functional inefficiency is mostly limited to the islets. In a few cases there is extensive disease of the whole gland (in dogs about nine tenths must be removed to cause diabetes), so that there is both diabetes and an inefficient external secretion. This comparatively small group has been classified separately by some writers as "true pancreatic diabetes."

With the exception of this group, therefore, the external secretion tests are of no value in diabetes mellitus. They are limited to the examination of diseases of the whole gland or its ducts.

### HYPERACTIVITY OF ISLETS

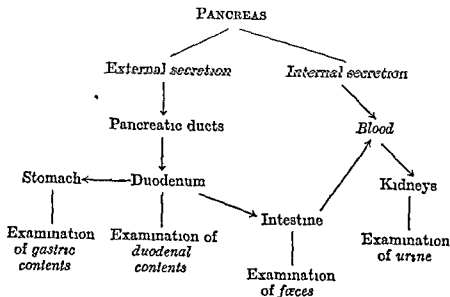
Reference has already been made (Chapter VII) to hyperinsulinism and new growths of the islets of Langerhans. Apart from hypoglycæmia these cases show no evidence of disturbance of pancreatic function.

### THE FUNCTIONS OF THE PANCREAS

I The formation of the internal secretion ("endogenous insulin")

II The formation of the external secretion (pancreatic juice), which contains ferments acting on each of the three classes of foodstuffs, viz amylase or diastase (carbohydrates), trypsin (proteins) and lipase or steapsin (fats).

It is difficult to examine these secretions because of the inaccessibility of the gland. This is obvious from the diagram given below, which also indicates the different fluids and excretions which may be utilised in making tests of pancreatic efficiency.



## TESTS OF A DEFICIENCY OF THE INTERNAL SECRETION

## Hyperglycæmia and Glycosuria

As already mentioned, glycosuria is uncommon in diseases involving the pancreas as a whole, and particularly in chronic lesions. In acute hæmorrhagic pancreatitis, slight transient glycosuria and hyperglycæmia are fairly often found, and are valuable in diagnosis, but there is rarely time to make estimations of blood sugar. In chronic diseases of the pancreas, it is worth while to obtain data for a blood sugar curve (Chapter VII), but the result is often within normal limits, because hyperglycæmia does not occur till a very large proportion of the islets is damaged. A normal curve therefore does not exclude pancreatic disease, and when an abnormal curve is obtained it is necessary to consider the possible influence of other factors (see Chapter VII). Having excluded the other factors, a diabetic type of curve indicates that the pancreatic lesion is very extensive.

## Loewi's Test

The pupils of both eyes are carefully examined, noting their size and their reaction to light and accommodation. Two drops of 1 in 1,000 adrenaline are instilled into one eye, the other being left untreated for comparison. The pupil of the treated eye is examined at intervals of about fifteen minutes for the next hour, and compared with that of the untreated eye. Normally the adrenaline causes no change in the pupil. The reaction is positive if the pupil dilates. In positive cases the pupil is often oval and the dilatation eccentric.

Several attempts to explain this observation have been made, of which two may be noted. The "hypoinsulinism" produces a relative hyperadrenalism which increases the sensitivity of the sympathetic system, wherefore the pupil reacts to the instillation of adrenaline. Inflammation of the pancreas causes irritation of the solar plexus, wherefore the autonomic system is hypersensitive.

The test is not specific because positive results have been obtained in hyperthyroidism, and in diseases of the gall bladder or of the bile duct. The latter observation might be "explained" on the basis of coincident pancreatic disease, but obviously limits the clinical value of the test. Negative results have been obtained in typical examples of acute pancreatitis. Some writers speak highly of the reaction in acute disease, others have discarded the test. It is so simple that it is worth performing in parallel with other tests, so long as its limitations are remembered. It is very rarely positive in diabetes mellitus.

## TESTS OF A DEFICIENCY OF THE EXTERNAL SECRETION

Theoretically, either tests might be applied for the different enzymes, or evidence might be sought of incomplete digestion of foodstuffs owing to deficiency of these enzymes. Examination of gastric contents, of duodenal contents, of fæces, of blood or of

urine, might be utilised. In practice many of these points of attack are of limited or no value, as will readily be understood if the presence of other enzymes in the alimentary tract, the action of bacteria, the influence of diarrhoea etc., are remembered. It is convenient to discuss the methods under the heading of each enzyme in turn.

### Carbohydrate-splitting Ferment

Examinations of gastric or duodenal contents are useless owing to the presence of salivary ptyalin. Examination of the feces has also proved disappointing, owing to the action of bacteria, diarrhoea, etc.

Estimation of diastase in the blood is of limited value. Normally, using Wohlgemuth's method, the blood contains 3 to 10 units of diastase (*cf* Harrison and Lawrence). In acute pancreatic disease, the amount is increased but owing to its rapid excretion, urine examination is much more satisfactory.

In the rare event of a lesion of the kidney accompanying pancreatic disease, the urinary diastase might not be significantly raised on account of the excretory failure of the kidney. If this combination should occur, it would be necessary to demonstrate that the high blood diastase was accompanied by a high normal or slightly raised diastatic index in the urine before it would be safe to ascribe the former to the pancreatic lesion, since the combination of a high blood with a low urinary diastase is well recognised in gross renal disease.

Diastase hydrolyses starch, yielding in sequence erythrodextrin, achroodextrin and maltose. In Wohlgemuth's test digestion is regarded as "complete" when no starch is left. At this point the mixture contains varying proportions of the products of hydrolysis. An alternative method (*cf* Myers and Killian) of estimating diastase in blood depends on blood sugar determinations without and with the addition of a starch solution to the blood, followed by incubation. This method gives quite a different end point from Wohlgemuth's, in that it measures the reducing sugars formed and not the disappearance of starch. The normal values obtained in this way lie between 15 and 25 per cent, *i.e.*, 15 to 25 per cent of the soluble starch employed (10 mgm) is changed into reducing sugars (calculated as glucose) in fifteen minutes at 40° C.

Estimation of diastase in urine has proved a useful test of pancreatic efficiency, although it is still not decided whether the diastase is derived solely from the pancreas. The technique of Wohlgemuth's method is given at the end of this chapter. The importance of adjusting the reaction (pH) of the urine to the optimum for the enzyme has been demonstrated by Dodds and by Sladden. Cohen and Dodds have introduced another method depending on the colorimetric estimation of the quantity of dextrin hydrolysed by the urinary diastase under standard conditions. A Wohlgemuth unit is the amount of diastase which will digest 1 c.c. of 0.1 per cent soluble starch (1 mgm) in thirty minutes at 37° or

38° C to such a degree that no blue colour is obtained on adding iodine. The diastatic index is the number of units in 1 c c of urine. In health the concentration of diastase in the urine, or the diastatic index, is 6.7 to 33.3 units. Occasionally when the urine is concentrated the index may rise as high as 50 units. The day's output of diastase is the product of the volume of the twenty-four hours' urine in cubic centimetres and the index. Normally, the day's output varies from 8,000 to 30,000 units.

An index of 50 is suspicious, and of 100 is almost always definitely abnormal. In acute pancreatitis values of 100, 200, and even up to 2,000 may be found. In chronic pancreatitis and in carcinoma of the pancreas the diastatic index is usually normal or low, but occasionally in carcinoma the index may rise to about 100. In severe trauma of the pancreas the urine not uncommonly contains an increased concentration of diastase (up to about 200 units), and the finding of high indices in the urines of patients who have had a blow in the abdomen is useful clinically. It has been claimed that the index is often high in the toxæmias of pregnancy, and it has been suggested that the cause of this is an increased permeability of the kidney. In many cases, however, the index is normal (though rarely, if ever, low). In eclampsia indices of 50 to 100 are fairly common, and very occasionally even higher values are obtained. It is possible that these figures are mainly, if not entirely, due to oliguria. In diabetes the diastatic index is almost always normal or low.

Estimations of diastase should be made on the twenty-four hours' urine, except in acute pancreatitis. Normally, owing to polyuria (drinking, etc.) or oliguria (sweating, etc.), there are considerable variations in the diastatic index of specimens taken at different times of the day, though this range of variation rarely exceeds 2 to 50 units. For this reason the twenty-four hours' sample is employed, and when this is done, the variations in different healthy individuals usually fall between 6.7 and 33.3 units, as already mentioned. In acute pancreatitis, diagnosis is so urgent that twenty-four hours cannot be allowed for collecting the urine, and the first obtainable sample is utilised. Fortunately, in genuine cases of the disease such a sample usually contains 100 or more units per 1 c c, but lesser concentrations of diastase are obviously not significant.

The increase of diastase in the blood and urine in pancreatic lesions may be due to obstruction of the ducts, or to increased permeability of the pancreatic cells due to inflammation. This hypothesis fits in with the clinical observation that the urinary diastase is increased when the ducts are obstructed or when inflammation is acute, and is normal or low in chronic sclerosing conditions. Cohen and Dodds have shown that the same variations in the urinary diastatic index are found in starvation as after food, so that presumably diet has no influence on the output of the enzyme.

In obstruction of a salivary duct or in acute disease of a salivary

gland, there may be a transient increase of the concentration of diastase in the urine (Kreyberg), but such a cause is readily recognised from the clinical examination of the patient

If there is a deficiency of the external secretion of the pancreas, it might be expected that a certain amount of starch might escape digestion and be recognisable in the fæces, but **microscopical examination of the stools for starch granules** has been found of no value as a test of pancreatic function. Starch granules are often absent in proved pancreatic disease, and are not uncommon when there is gross diarrhoea or the subject is ingesting large quantities of starchy foods

### Protein-splitting Ferment

Either the gastric or the duodenal contents may be examined for trypsin. A positive result excludes complete obstruction of the pancreatic ducts but a negative result in either fluid is not conclusive. It is unlikely that quantitative estimations will be of any value owing to the unknown degree of dilution by bile, etc. *Regurgitation of duodenal fluid into the stomach is uncertain* (cf Chapter XXII), but it has been suggested that the reflux might be provoked by administering 0.4 per cent hydrochloric acid. Generally, however, direct duodenal intubation is employed (see Chapter XXIII). When examining the gastric contents for trypsin it is of course, essential to make the reaction alkaline.

Examination of the fæces for protein-splitting ferments has proved disappointing. In a certain number of healthy individuals no such enzyme can be detected and in the rest, when quantitative methods are employed and the results are expressed in terms of the dried fæces, a wide range of variation is obtained. It would appear therefore that results characteristic of pancreatic lesions do not exist. A simple test for the detection of trypsin in fæces, duodenal or gastric contents, etc., is given in Chapter XXIV.

Examination of the blood and urine for trypsin is of little or no value.

If the external secretion of the pancreas is deficient, it might be expected that the fæces would contain more protein and protein products than normally, though the action of intestinal erepsin, and of bacteria, and the influence of the rate of passage of the intestinal contents are all obvious complicating factors. Whilst it is true that the total nitrogen expressed as a percentage of the dried fæces is increased in gross pancreatic disease, such a wide range of variations is found in other pathological conditions—gastro-intestinal disease often giving quite as high values as pancreatic disease—that the estimation is of little or no value. A proper metabolism experiment in which the intake and output of nitrogen are measured gives more information, but is not a practical proposition in routine clinical work.

Microscopical examination of the fæces for undigested muscle fibres is a simple test of some value but it is important to realise



what constitutes "creatorrhœa," or the passage of undigested meat fibres. In Fig 51 are shown the different stages in their digestion. The connective tissue binding the fibres together is digested by the gastric juice, so that pancreatic trypsin acts on individual muscle fibre fragments. There results *seriatim* the disappearance of the nuclei, the rounding of the ends of each fragment, and the disappearance of the transverse striations, leaving round yellow brown fragments, which may either show a

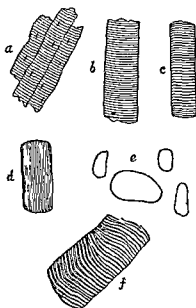


FIG 51 Undigested and partially digested muscle fibres as seen in faeces. *a* Mass of fibres not digested at all. Note irregular ends, nuclei, and transverse striations. *b* Isolated fibre undigested. Note irregular ends and transverse striations. *c* Partially digested fibre. Note transverse striations but rounded ends. *d* Fibre still more digested than *c*. Note rounded ends and faint longitudinal striations. *e* Fragments of fibres free from striations and with rounded ends. *d* Digestion more complete than in *d*. *f* Woody fibre from vegetable.

faint longitudinal striation or may appear quite homogeneous, and finally the complete disappearance of muscle fibres. Fibres as depicted at *c*, *d*, and *e*, in Fig 51, may be seen in normal stools. Fibres with rounded ends but visible transverse striations (Fig 51, *c*) are not uncommon in simple diarrhœa. The discovery of numerous fibres with irregular ends and well marked striations, "true creatorrhœa" (Fig 51, *a*, *b*), almost always points to a deficiency of pancreatic trypsin, but their absence does not exclude a gross lesion of the pancreas, since other protein splitting ferments (intestinal or bacterial) may cause partial or complete digestion of muscle. The only things likely to be mistaken for muscle fibres are the woody fibres of certain vegetables (Fig 51, *f*). There is rarely any serious difficulty, however, because the striations of the woody fibres are much coarser, more irregular, and more refractile. Naturally, when the observations are made, the patient must be consuming meat or poultry. Some investigators have claimed that better results may be obtained with special test diets, but for

routine clinical work an ordinary hospital diet, containing meat, is generally satisfactory. For the examination all that is necessary is the emulsification of a platinum loopful of faeces in a drop or two of physiological saline on a slide. Several preparations should be made, mounted under cover slips and examined with a one sixth objective. With a little practice it is easy to avoid making the emulsion so thick that it is impossible to see through it, or unnecessarily thin.

### Fat-splitting Ferment

Tests for lipase in gastric contents, blood, or urine, are of little or no value clinically (see Chapter XII for remarks on blood lipase). Coope recommends the technique of Carnot and Mauban for the detection of lipase in duodenal contents.

Mix a 2 per cent agar agar solution with an equal volume of 5 per cent starch paste and about one fortieth of its volume of neutral fat (e.g. pure fresh butter or lard). Heat stirring carefully till a homogeneous emulsion is produced. Add a trace of bile salts, pour into a clean Petri dish and cool rapidly.

Distribute small drops of the duodenal fluid over the surface of the medium with a fine pipette. Incubate at 38° C for one hour. Cover the surface with saturated copper nitrate for ten minutes. Rinse carefully with water. If lipase is present, tiny patches of fatty acids transformed into copper soaps are seen as bluish green specks standing out against the white background provided by the starch in the medium.

As in the case of tests for trypsin in duodenal fluid, a positive result excludes a complete obstruction of the pancreatic ducts, but a negative result is not conclusive evidence of the presence of such obstruction. Tests for the two ferments should be run in parallel.

Tests for lipase in the faeces are of no value, because the lipase is destroyed by trypsin, but microscopical examination of the stools and a differential estimation of fat in faeces (see Chapter XXIV for technique) are generally recognised as of value in pancreatic disease.

### General Appearance and Microscopical Examination of Faeces

The passage of bulky stools is a well recognised clinical feature of pancreatic disease, though other conditions may cause bulky stools, e.g., obstruction of the bile duct, coeliac disease, sprue, congenital steatorrhoea, gastro colic fistula and mesenteric tuberculosis involving the lacteals. Bulky, watery stools, due to diarrhoea, are easily differentiated. The stools in the above diseases are not only bulky, but fatty in appearance, owing to the presence of an excess of fat. The passage of fatty stools, which are liquid when passed and set solid on cooling, constitutes "true steatorrhoea," though the term steatorrhoea is often applied to the passage of any fatty stool, even though it does not solidify on cooling. True steatorrhoea is apparently diagnostic of pancreatic defect (except possibly in congenital steatorrhoea). The fresh stool consists of faecal material floating in molten oil, and on cooling the faecal matter forms a plate.

Microscopical examination is useful, not only to check the macroscopical appearance of fat, but also to give a rough idea of the type of fat (neutral fat globules, fatty acid crystals, soap plaques) present. Fuller details are given in Chapter XXIV. Here a few points with special reference to pancreatic disease only need be mentioned. When there is a deficiency of the pancreatic juice, it is the splitting of the fats which is defective. Hence the excess of fat is chiefly in the form of neutral fat. On microscopical examination, therefore, only globules, but not an excess of fatty

acid crystals or of soap plaques, may be found in a typical uncomplicated case. In clinical work, however, cases of combined pancreatic and liver disease are commoner than pure pancreatic disease, wherefore the microscopical findings are often not "typical." Moreover, the neutral fat is often so finely divided that it is not readily seen under the microscope, and when only globules are visible it is important to exclude liquid paraffin and other oily drugs as their source. The results of microscopical examination therefore can only be interpreted in the light of the clinical examination. Lastly, even in the complete absence of pancreatic juice, the neutral fats may be hydrolysed lower in the gut by bacteria, for which reason the absence of a "typical" microscopical picture by no means excludes the existence of pancreatic disease.

In the laboratory, one of the chief reasons for making a microscopical examination is to decide whether or not the laborious analysis of faeces for fat is worth undertaking. If macroscopically, the faeces are not bulky and not fatty looking, and if microscopically no fat globules, no fatty acid crystals, and only occasional soap plaques are seen, then chemical analysis is certainly a waste of time.

#### Fat in Faeces

The technique is given in Chapter XXIV. Normally, of the dried faeces, not more than one fourth (or one third in children—see p. 463) is fat, and of the faecal fat not more than one fourth (or one third in children) is unsplit. In typical pancreatic cases, the total fat percentage is excessive, often over 50, and the splitting is defective, the unsplit neutral fat being 40 to 70 per cent, or even more, of the faecal fat. But as indicated in the last section, such typical results are the exception rather than the rule, and the finding of a high percentage of total fat of which the greater proportion is split does not by any means exclude pancreatic disease. Differentiation between free fatty acids and soaps is of no value, since the reaction of the faeces is the chief factor influencing the proportion of fatty acids which is combined as soaps. In view of all these points the writer would limit fat analyses to those cases where there is obviously an excess of fat on macroscopical and microscopical examination, and to special investigations, for the work is time consuming.

The table on p. 275 summarises the results of the tests considered above —

#### OTHER TESTS IN PANCREATIC DISEASE

Several attempts have been made to obtain evidence of a deficiency of the external secretion of the pancreas by administering artificial preparations. The reader is referred to Coope's book for references. In Sahli's glutoid capsule test, drugs such as iodoform, methylene blue, or salol, easily recognised in the urine or saliva, are administered in gelatin capsules hardened by formalin so that

*The Results of Tests of Pancreatic Efficiency*

Condition	Loevis test	Glycosuria	Hyperglycemia	Urinary diastase units per 1 c.c.	Trypsin in duodenal fluid	Creato ribose	Lipase in duodenal fluid	Stearo ribose	Fat in faeces	
									Total fat (per cent of dried faeces)	Split fat (per cent of faecal fat)
Health	0	0	0	6.7 to 33.3	+	0	+	0	Up to 75	75 or over
Acute pancreatitis	Often +	Often +	Often +	100 to ∞	—	0 or +	—	—	—	—
Trauma of pancreas	0 or +	0 or +	0 or +	6.7 to 200	—	0	—	—	—	—
Chronic pancreatitis	Usually 0	Usually 0	Usually 0	Usually normal	+ or ±	+ or ±	+ or ±	0 to +	Normal or raised	Normal or less than 75
New growth of pancreas	0	0	Usually 0	6.7 to 100	+ or 0	+	+	0	Usually over 25 up to 50 or more	Usually below 75 or less
Chronic obstruction of pancreatic duct	0	0	0	6.7 to 100	0	+	0	0	Normal	Normal
Diabetes mellitus (excluding true pancreatic)	0	+	+	Normal or low	+	0	+	0	Usually over 25 up to 50 or more	Usually below 75 or less
New growth of islets	0	0	Hypo glycemia	Normal	+	0	+	0	Normal	Normal

0 signifies negative. The 4 hours urine except in acute pancreatitis — not known with certainty ± present but diminished  
 + positive + + true steatorrhea — not known with certainty ± present but diminished  
 † Figures refer to adults

they will escape digestion in the stomach but will be digested in the intestine if pancreatic juice is present. In Schmidt's beef cube test, small cubes of beef are hardened in alcohol placed in bags of silk gauze, ingested and finally recovered from the faeces. They are then examined to see whether the nuclei are digested. In Winternitz's sajodin test, the calcium salt of iodo behenic acid (sajodin) is ingested and the urine is examined for iodide. The sajodin is hydrolysed only by pancreatic juice, and only in the

presence of bile salts, so that failure to detect iodide in the urine indicates pancreatic inefficiency, provided there is not obstruction to the bile passages. The general opinion of these tests is that they are of little or no value in clinical work—an opinion with which the writer agrees from his own observations. A similar conclusion applies to Cammidge's test, which has fallen into disuse.

Calcium oxalate crystals have been observed in the urines of patients with pancreatic disease, as in many other conditions, and there is nothing to indicate that they have any special connection with pancreatic lesions. Indicanuria has likewise been selected for notice in pancreatic disease, but whilst it is reasonable to assume that when there is a deficiency of protein splitting ferment, there will be greater opportunities for the putrefaction of proteins by bacteria in the large intestine, there is no evidence that tests for indican are of any special value in suspected disease of the pancreas.

A similar attitude is justified in regard to tests for occult blood in faeces. A positive reaction indicates hæmorrhage somewhere in the alimentary tract. If clinically there is a carcinoma of the head of the pancreas and blood is found in the faeces there is a presumption but no proof, that the growth is ulcerating into the bowel.

Wallis has claimed that the percentage of ash from the dried faeces is low when there is obstruction of both pancreatic and bile ducts.

Dodds and his colleagues (see Chapter XXII) have claimed that the alveolar  $\text{CO}_2$  rises during the secretion of gastric HCl and falls during the secretion of pancreatic juice, but the possibility of utilising this observation as an index of the output of pancreatic juice has not been explored. In any case the technique is too complicated to be used in routine clinical work.

## SUMMARY

It is convenient to summarise the tests of most value under the headings of the different fluids or excreta examined. Loewi's is a purely clinical test.

**Urine.** Routine chemical examination (see Chapter II), and particularly tests for sugar.

Estimation of *diastase* in the twenty four hours' urine, except in acute pancreatitis and operative emergencies, when the first specimen obtainable is employed.

**Blood.** Estimation of *blood sugar*. Data for a blood sugar curve (see Chapter VII) in special cases. Estimation of *diastase* on occasion (usually urinary *diastase* sufficient).

**Fæces.** Naked-eye appearance—*bulk*—*steatorrhœa*.

Microscopical examination for *undigested muscle fibres*, *fat globules*, *fatty acid crystals*, and *soap plaques*.

*Differential estimation of fat*.

**Duodenal Contents.** Tests for *trypsin* and *lipase*.

## CONCLUSION

In *acute* lesions of the pancreas the function tests are often valuable. In the limited time available, Loewi's reaction, examination of the urine (glycosuria) and an estimation of urinary diastase can readily be performed.

In *chronic* lesions the tests are often disappointing. If two or three give positive results, the existence of pancreatic disease is likely. If all give negative results, pancreatic disease is not excluded. An examination of the urine, including an estimation of diastase, should be made as a routine. The naked eye appearance and microscopical examination of the faeces likewise should always be undertaken, but other tests should be reserved as an extension of particular clinical observations and for special investigations.

## TECHNIQUE FOR ESTIMATION OF DIASTASE (AMYLASE) IN URINE

## Principle

A known quantity of soluble starch is digested with varying amounts of urine for thirty minutes at  $37^{\circ}\text{C}$ . After immediate cooling iodine is added to determine the smallest quantity of urine that has completely digested the starch, to a point at which it no longer gives a blue colour.

## Solutions

*Stock solution of soluble starch* (2 per cent starch in 10 per cent sodium chloride)

Weigh out accurately 2 gm. of pure soluble starch, and make a fine emulsion by shaking it thoroughly with 10 c.c. of distilled water in a test tube. Add the emulsion to about 70 c.c. of boiling distilled water, washing out the tube with a few c.c. of water three or four times, and adding the washings to the boiling mixture. Then add 10 gm. of pure sodium chloride. Boil and stir the mixture well. Cool, transfer to a 100 c.c. volumetric flask quantitatively, and make up to volume. Add a few drops of toluene as preservative.

*0.1 per cent soluble starch in 0.5 per cent sodium chloride*

Transfer 5 c.c. of the stock starch solution to a 100 c.c. volumetric flask, and make up to the mark with distilled water. If a few drops of toluene are added the solution will keep for a week.

*N/50 iodine solution*

This is prepared freshly by diluting 2 c.c. of N/10 iodine (see Appendix) with 8 c.c. of distilled water, and keeps only for a few days.

## Method

*Adjust the reaction* of the urine as follows. Take the reaction to litmus paper, and accordingly add either 40 per cent sodium hydroxide or concentrated hydrochloric acid drop by drop to the main bulk of the urine (200 c.c. or more) until the reaction is neutral.

or very slightly acid. By this means the dilution is generally negligible. If, however, only a small quantity of urine is available, adjust the reaction of a known volume with N/10 NaOH or N/10 HCl from a burette. Note the volume of alkali or acid required, and allow for the dilution in the calculation.

*Prepare a series of dilutions of urine as follows, plugging lightly the ends of all pipettes with cotton wool to prevent contamination with salivary diastase.*

In tubes (3 in  $\times \frac{5}{8}$  in are suitable) numbered 1, 2, 3, etc., place 1.0, 0.6, 0.4, 0.3, 0.25, 0.2, 0.15, and 0.1 c.c. of well mixed urine. Make the contents of each tube up to 1 c.c. with distilled water. Add 2 c.c. of the 0.1 per cent. starch to each tube, and mix well. Incubate for thirty minutes at 37° C. Cool immediately by placing the rack of tubes in a basin of cold water for three or four minutes. To each tube in turn add N/50 iodine drop by drop, continuing the addition until it is clear that a blue colour is or is not formed. It is important to add the iodine carefully in this way. When the starch is scarcely digested the blue is obvious and cannot be masked by an excess of iodine. Likewise when all the starch has gone, an excess of iodine does not matter and merely intensifies the yellow brown tint. When, however, the tube contains a little unaltered soluble starch and much erythrodextrin, it is easy to mask the trace of blue due to starch by the red due to erythrodextrin, if an excess of iodine is added.

Note the tube containing the least amount of urine which gives no blue colour with iodine. The quantity of urine in this tube is just sufficient to digest the starch to the stage defined.

If 0.1 c.c. of urine completely digests 2 c.c. of the starch solution, dilute the urine 1 in 10 with distilled water, and put up a second series. On rare occasions (diastatic index greater than 200) the urine has to be diluted 1 in 100.

### Calculation

Suppose that the tube in which the digestion of the 2 c.c. of starch is just "complete" (as defined), contains  $x$  c.c. of urine.

Since a unit of diastase is that amount which just digests 1 c.c. of 0.1 per cent. soluble starch at 37° C. in thirty minutes to such a degree that no blue colour is obtained on adding iodine, therefore  $x$  c.c. of urine contains 2 units of diastase.

1 c.c. of urine contains  $\frac{2}{x}$  units of diastase, or otherwise expressed, the diastatic index, or the number of units in 1 c.c. of urine, or  $d \frac{37^\circ}{30} = \frac{2}{x}$ .

For example, 0.4 c.c. of urine just digested 2 c.c. of the starch solution,

$$\therefore d \frac{37^\circ}{30} = \frac{2}{0.4} = 5 \text{ units}$$

The tables given below are useful in putting up the series, and

indicate the diastatic index for each tube when digestion is just complete in that tube

Tube	1	2	3	4	5	6	7	8	
c c of urine	10	06	04	03	025	0°	015	01	Add 2 c c of 0.1 per cent starch to each tube
c c of water	00	04	06	07	075	08	085	09	
$d\frac{3}{30}$	20	33	50	67	80	100	133	∞00	

Tube	1	2	3	4	5	6	7	8	9	10	11	
c c of 1/10 urine	1	085	07	06	05	04	03	0°	015	01°	01	Add 2 c c of 0.1 percent starch to each tube
c c of water	0	015	03	04	05	06	07	08	085	088	09	
$d\frac{3}{30}$	∞0	35	∞86	333	400	500	66	100	1333	1667	∞0	

An example of the calculation of the days output of diastase follows —

Diastatic index or units of diastase per 1 c c was 10 Volume of twenty four hours urine was 1 400 c c

Days output of diastase =  $10 \times 1,400 = 14\,000$  units

### Notes

(1) *Adjustment of Reaction* Sladden and Dodds have introduced more accurate methods for adjusting the reaction to pH 6.7 (optimum for diastase in chloride solution) or to pH 6.1 (optimum for diastase in phosphate solution) Stafford and Addis find that the optimum reaction for the enzyme in a mixture of chlorides plus phosphates (i.e. in urine) lies between pH 6.1 and 6.5 In all published work the results obtained with urines whose reactions are close to the optimum are little if at all different from those with urines accurately adjusted It is clear, therefore that the routine technique adopted above is not only simple but also reasonably accurate

(2) As mentioned in the text, a sample of the *twenty four hours' urine* preserved with toluene should be employed except in emergencies (acute pancreatitis)

(3) It is very important to prevent *contamination with salivary diastase* by protecting with cotton wool the ends of all pipettes placed in the mouth

(4) Some workers after making the dilutions of urine, place the rack of tubes and the flask of soluble starch in the water bath at 37° C for a few minutes in order to bring their temperature up to 37° C *before* adding the 2 c c of starch to each tube Thus results in very slightly increased diastatic values and theoretically would be the best method if everybody did the same, in that it obviates slight variations due to the different temperatures of different laboratories In practice it is unnecessary The contents of the



tubes reach 37° C in two or three minutes in a water bath (and not quite so quickly in an incubator) Incidentally a pipette calibrated to deliver 2 gm. of water at 18° C. will deliver very slightly less at 37° C These and other minor points of refinement make no significant differences

(5) It is important to *shake the urine well* before taking samples, because an appreciable amount of enzyme may be adsorbed on a deposit

(6) A similar technique may be applied to serum or plasma for the *estimation of blood diastase*, though, of course, no preliminary adjustment of reaction is necessary Venepuncture is required to obtain 3 or 4 c c of plasma Alternatively, when little serum or plasma is available, a drop method may be employed

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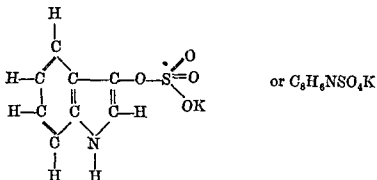
## CHAPTER XIV

### INDICANURIA. MISCELLANEOUS URINE TESTS

[Most of the information collected together in this chapter is scattered through the books on physiological chemistry, clinical diagnosis, chemical pathology, etc., to which reference has been made in previous chapters.]

#### INDICAN

SO CALLED primary indican is potassium indoxyl sulphate. At least the salt is always represented as of potassium, though possibly indoxyl sulphuric acid is partly combined with sodium or other bases in urine.



True indican is a vegetable glucoside, and is the source of the natural indigo obtained from plants.

Indicanuria results from the bacterial decomposition of tryptophan. This decomposition almost always occurs in the large intestine, though occasionally in decomposing pus anywhere in the body (e.g., gangrene of the lung, empyema, etc.). Indole is formed by the bacterial action, and some of it is absorbed and carried in the portal blood to the liver, where it is oxidised to indoxyl and conjugated with sulphate. The resulting indican passes into the general circulation and is very rapidly excreted by the kidneys. The concentration in the blood is minute except when there is gross renal disease (cf. Chapter V). Most of the indole is excreted in the feces. In the majority of cases, therefore, the following factors influence indicanuria, viz., the amount of protein (tryptophan) in the diet, the bacterial flora of the intestine, the opportunity for bacterial action on protein or protein products (stasis, constipation, deficiency of protein-splitting enzymes, etc.), and the efficiency of detoxication by the liver.

Indican is an ethereal sulphate and may be taken as representative of the toxic putrefactive bodies (skatole, paracresol,

phenol, etc.), which are rendered innocuous by the liver or by other tissues. Alternatively, indoxyl may be conjugated with glycuronic acid, and excreted as indoxyl glycuronate (*cf* Chapter VI). The degree of indicanuria is thus a rough measure of the amount of decomposition going on in the intestine, provided the other factors mentioned above are not more important.

Normally the urine contains only traces of indican, 0 to 32 usually 5 to 20 mgm in twenty four hours (Borden), but simple constipation may cause a considerable increase. In disease, therefore, only gross and persistent indicanuria is of significance. The influences of diseases of the liver (Chapter XII) and of the pancreas (Chapter XIII) have already been discussed, as also the formation of indigo blue *in vitro* when the urine is exposed to air (Chapter IV). In skin diseases tests for indican have been regarded as valuable as pointing to those cases in which the combination of local treatment and of intestinal antiseptics is likely to be advantageous. Some of the most marked examples of indicanuria have been encountered in intestinal ulceration (*e.g.*, tuberculous, typhoid, etc.) and in peritonitis. When testing urines rich in indican, the extracted urine often turns deep brown owing partly to the formation of pigments from other chromogens, and partly (possibly) to oxidation products of indoxyl other than indigo blue. The urine of healthy breast fed infants is often indican free. Darkening of indican rich urine may even occur spontaneously on exposure to air, and has been mistaken for melanuria.

Simple qualitative tests (Obermayer's, Jaffe's) for indican have already been described in Chapter II. They both depend on the liberation of indoxyl from indican by acid, and the oxidation (and condensation) of indoxyl to indigo blue, which is extracted by chloroform (or ether or amyl alcohol), and recognised by its colour and absorption band in the red (see Fig 48 facing p 220). Care is necessary to avoid excess of oxidising agent which oxidises indoxyl beyond the indigo blue stage to a colourless compound. If the urine to be examined contains iodides (Chapter XI), iodine is liberated and extracted by the chloroform, and may mask traces of indigo blue. If a few drops of sodium thiosulphate solution (about 10 per cent) are added, and the tube is shaken well, the iodine is decolourised, but the indigo blue is unaffected. Skatole red and indigo red (see below) are not decolourised by "thio".

Place in a boiling tube about 5 c.c. of urine, 6 c.c. of concentrated HCl, 3 or 4 c.c. of chloroform and a few drops of 10 per cent sodium thiosulphate. Mix well by pouring into another tube and back repeatedly. Allow the chloroform to separate and examine its colour. Then add 1 drop of 1 per cent potassium chlorate and mix thoroughly again. Repeat the process adding 1 drop of the chlorate solution each time till it is clear whether indigo blue is formed or not. By this means iodine is decolourised as fast as it is formed and traces of indigo blue will not be missed through over oxidation.

Jolles has shown that the addition of thymol makes the above reactions more sensitive. The simplest method is to add a pinch of powdered thymol to the urine in the test tube, and then proceed in

the usual way with Jaffe's or Obermayer's test. The colour of the chloroform extract is then purple blue and not pure indigo blue.

Andrewes' diazo test for indican in blood (see Chapter V) cannot readily be applied to urine.

For quantitative determinations the reader is referred to Borden's paper and to larger works.

### SKATOLE-RED

Skatole red is a substance of unknown constitution. It is formed under the same conditions as is indigo blue from indoxyl-sulphuric acid, viz., treatment with acid oxidising agents, and the significance of the two is the same. Skatole red, however, is not so readily detected as indigo blue, partly owing to its colour, partly owing to its lesser concentration, and partly owing to the difficulty of differentiating it from other reddish pigments (e.g., urochrome, Chapter XI). Tests for the unknown precursor (possibly skatole, or a related substance) of skatole red have not been widely employed or greatly studied in clinical work. To complicate matters still further some indican is oxidised to isatin, which condenses with unchanged indoxyl to form indigo red (indirubin). Skatole red is insoluble in chloroform and ether, and its colour is unaltered by sodium thiosulphate whereas iodine (from iodides) is decolourised. Indigo red is soluble in chloroform and ether, whilst both indigo red and skatole red are soluble in amyl alcohol. There has been considerable confusion between indigo red (indirubin or urochrome), skatole red, and urochrome (formed from indole acetic acid (skatole-carboxylic acid) or indole acetic acid by adding HCl), and the subject needs further investigation. Homer has made a careful study of the absorption spectra of the colour reactions of certain indole derivatives, including the above.

Fearon and Thompson also have discussed this group of red pigments, and have described another reaction, the urochrome reaction, which they regard as due to condensation between indoxyl and o- or m-cresol or other phenolic compounds.

### EHRlich's DIAZO TEST

#### *Solutions*

(a) A saturated or 0.1 per cent solution of sulphuric acid in 5 per cent v/v hydrochloric acid.

(b) Half per cent aqueous sodium nitrite.

Prepare the diazo reagent on the day of the test by mixing 50 parts of (a) and 1 part of (b).

#### *Method*

To about 5 c.c. of urine add an equal volume of the diazo reagent and shake well to mix. Add 1 c.c. or more of concentrated ammonia so that the reaction becomes strongly alkaline, and shake again. Note any colour change.

*Results*

Normally a yellow colour is observed. In typhoid fever the urine turns red and the froth also shows a reddish tinge. This reaction has been observed from the fifth to the twenty second day. Positive results have also been reported in measles, german measles, acute tuberculosis and other fevers. Some workers have attached considerable value to the test in typhoid fever. Others find it of little value in the diagnosis of that disease. Von Jaksch says "No chemist would consent to draw any conclusions from a reaction so long as he is in complete ignorance of the substance or substances to which it is due. Only the medical man who lacks the necessary chemical training is prepared to do this." This view is possibly an extreme one, but of late years the reaction appears largely to have fallen into disuse.

The method is very similar to Andrewes' test for indican in blood (see Chapter V). A more acid and a larger proportion of diazo reagent is used in Ehrlich's test, but the important differences from Andrewes' reaction are that, in Ehrlich's test, the diazotised mixture is not boiled, and that ammonia and not caustic soda is used to make the mixture alkaline. The writer has failed to obtain positive Ehrlich's reactions with normal urine to which has been added either potassium indoxyl sulphate or N acetyl indoxyl to the extent of 40 mgm per 100 cc or more. The substance responsible for Ehrlich's test is, therefore, not indican. The cause of the reaction is still unknown. For further observations, including references, the reader should consult Hunter's paper.

**CHYLURIA**

The occurrence of opaque white milky urine is known as chyluria. Its artificial production by the deliberate addition of milk or of condensed milk must be excluded! An opalescence due to large numbers of bacteria, with or without a phosphatic scum, or due to added oily preservatives (toluene, chloroform, etc) is easily recognised.

True chyluria, as the term implies is due to chyle of the intestinal lymphatics gaining access to the urine. Chyle contains among other things fats, proteins including fibrinogen and a few red blood corpuscles, the fats are very finely divided, the droplets being visible only under a  $\frac{1}{2}$  in objective with dark ground illumination. The diagnosis of chyluria therefore depends on showing that the urine contains fat, and that this fat is not visible as droplets under the  $\frac{1}{2}$  in objective with ordinary lighting, also that erythrocytes and fibrin clots are present, though the clots are easily missed when the proportion of chyle is low. When the proteins are precipitated the fat is often also carried down. The opalescence may appear to be unaffected by simple extraction with ether in a test tube, owing to replacement of the opacity due to fat by another emulsion. Quantitative measurements, employing repeated extraction with

ether, have revealed traces to about 3 per cent of fatty matter. An excess of cholesterol in the urine has also been reported. In the clots are embedded epithelial cells and varying quantities of red cells and leucocytes. In marked chyluria, the urine has a reddish tinge (hæmatochyluria), and the clot may be slightly red owing to the entangled erythrocytes.

Chyluria is uncommon, often intermittent and usually unaccompanied by any serious departure from health. It is due to rupture of varicose lymphatics in the wall of the bladder or pelvis of the kidney. The distension of the lymphatics is due to obstruction, the most common cause being filarial parasites. Some of the patients, however, have never been in the tropics, their lymphatic obstruction is generally due to tuberculous fibrosis subsequent to peritonitis or abscess.

As would be expected, the milkiness of the urine varies with the intake of fat in true chyluria, and one way of showing that there is a fistulous connection between the lymphatic and urinary systems is to administer by mouth an unsaturated fat with a high iodine value, and to show that the ether extract of the urine similarly has a raised iodine value. A simpler method, however, is to give by mouth 100 mgm of Sudan III in 10 gm of butter (*e.g.*, in a lettuce sandwich) and to extract the urine with ether, in positive cases the ether extract is red.

Dr H. Morley Fletcher reported a non-parasitic case so tested, before swallowing the dye the ether extract of 25 c.c. of the urine was yellow; after the dye the same volume of urine yielded a red extract in the third and up to the thirteenth or fourteenth hours—see frontispiece for an illustration of the result of one of these experiments. In this case though the urine was often very milky, the fat lost by this channel was insignificant and of the order of 1 to 3 gm daily. Mr W. Girling Ball obtained the urine from each kidney by ureteric catheterisation, both samples were free from protein and were not milky. Cystoscopically he observed just below the right ureteric orifice a minute hole from which milky fluid was exuding. At laparotomy he found definite evidence of an old peritonitis but no signs anywhere in the abdomen of a gross obstruction of the lymphatics, which, however, were obviously dilated particularly on either side of the bladder. He closed successfully the fistulous opening into the bladder by cauterising but later there was a fresh rupture but into the reproductive tract and almost pure milky chyle escaped from the vagina.

Green Arnytage has reported a case of 'metrochylorrhœa' or 'intermittent chylous metrorrhœa' in which there was a milky chylous discharge from the uterus; Sudan III, given orally dyed the discharge, there was no chyluria.

The Sudan III test has also been carried out in filarial chyluria (personal communication, N. H. Fairley and R. J. Bromfield), in some cases the deflection of chyle to the urine was so marked that the urine was obviously red when passed, and ether extraction was unnecessary except to show that the red pigment was taken out by a fat solvent.

### LIPURIA

This term is applied when fat is present in the urine, and therefore should include chyluria. In non-chylous lipuria the fat is

usually not so finely divided as in chyluria, and is generally visible under the microscope with a  $\frac{1}{8}$  in objective and ordinary lighting. Moreover, in lipuria, the urine, though opalescent, is not usually so milky in appearance.

It is important to exclude extraneous fat as responsible for "lipuria," *eg*, the lubricant used for catheters, contamination with liquid paraffin from the rectum or with oily substances used for intraurethral medication. The rupture of a cyst into the urinary tract may be responsible for temporary "lipuria."

The writer is doubtful whether true lipuria exists apart from chyluria but it has been reported from the ingestion of large amounts of fats, the subcutaneous injection of much oil, and extensive oilyunctions. It has also been observed in diabetes mellitus with gross hæmia, after tearing or crushing of the subcutaneous fat after fractures of long bones, in chronic nephritis with advanced fatty changes in the kidneys, in phosphorus poisoning, in eclampsia, and in other conditions. In some of the reported cases, however, sufficient care to exclude extraneous fat, and to prove that the opalescence really is due to fat, does not seem to have been taken.

The fat should be extracted with ether or other suitable solvents, quantitative measurements being made whenever possible. For the technique of extraction and for further information as to methods for identifying the type of fat extracted, the reader is referred to larger works.

### NITRITURIA

Nitrituria is common in infections of the urinary tract by organisms which can reduce nitrates (*B. coli*, staphylococci, *B. proteus*, *B. lactis aerogenes* etc.) and absent in infections due to streptococci, gonococci, *B. tuberculosis*, etc., which do not form nitrites. Indeed, Weltmann has suggested that tests for nitrites might be useful as evidence of such infections when microscopical and bacteriological examinations are not available. Fresh normal urine does not contain nitrites, but about  $\frac{1}{2}$  gm of nitrates per diem.

Greenthal found the nitrite test positive in 5 out of 10 cases of pyuria in children, though it is noteworthy that in only one of the positive cases was the urine obtained by catheter. He suggests that failure of the nitrite test in pyuria (due to organisms of the colon group) is due chiefly to lack of nitrates in the urine.

In the writer's opinion it must seldom happen that the chemical test is available when microscopical and bacteriological examinations are not, and every physician ought to use his microscope for simple examinations such as the detection of pus in urine. It is admitted that no chemical test provides so certain a diagnosis of pyuria as does the microscope, wherefore the chemical tests would seem to be superfluous. For this reason the reader is referred to the articles cited and to other books for methods of testing for nitrites.

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## CHAPTER XV

### CHLORIDES. THE INORGANIC CONSTITUENTS OF THE URINE

A QUALITATIVE test for chlorides in urine has been given in Chapter II. For quantitative methods the reader is referred to the standard works on physiological chemistry.

For clinical purposes qualitative tests generally give all the information required, since only a grossly deficient excretion of chlorides is of any significance. Both in health and in disease, the intake of chlorides influences greatly the excretion in the urine, and metabolism experiments are essential in order to interpret results slightly above or below the range of excretion in health. Quantitative analyses are therefore practically limited to metabolism experiments, which are themselves limited to special investigations (see also Chapter V).

In clinical work therefore, we are concerned only with a gross fall in the output of chlorides such as is revealed by qualitative methods. The low output of chlorides in nephritis has been discussed in Chapter V, where it was pointed out that chlorides are "locked up" in cedema fluid and therefore there is less to be excreted by the kidney. Whenever there is extensive cedema, whether or not it be associated with renal disease, there is a low output of chlorides. Furthermore, all extensive collections of fluid in the body tend to cause 'chloride retention,' and whenever there is a gross loss of fluid other than through the kidneys, the output of chlorides in the urine tends to fall. Thus in pneumonia, large quantities of fluid are transported to the lungs, and are deposited in the pneumonic exudate. This fluid contains chlorides, for wherever there is retention of water, there must be retention of salt also to maintain osmotic equilibrium, and so in pneumonia an actual fall in the blood chloride results with a consequent gross drop in chloride excretion. Similarly in ascites, in peritonitis when the exudate is great, and in large empyemata, there is a diminished excretion of chlorides in the urine. The loss of chlorides by vomiting, the consequent fall in blood chloride, and therefore of urinary chloride, and the accompanying alkalosis has already been described in Chapter IX. There also was mentioned the importance of making good the fall in blood chloride in intestinal obstruction. Of recent years, owing to the resort to blood analysis in the first place, the value of urine examination has tended to fade into the background. Whilst it is true that the interpretation of qualitative tests for chloride in the urine may be complicated by oliguria, polyuria, or the difficulties of collection of urine, the urine test is exceedingly simple and takes only a few minutes.

The urine test is probably of most value in the diagnosis of pneumonia, and especially in children, in whom the physical signs in the chest are often slow in development. A low concentration of chloride in the urine is of course not limited to this disease, but may be a useful finding as an extension of the clinical examination. At the crisis there is a sudden return of chlorides to the urine. In extensive burns, the urinary chloride may be low, owing to the great loss of fluid from the damaged surface, and saline injections form an important part of the treatment.

### OTHER INORGANIC CONSTITUENTS

*Phosphates* and "phosphaturia" have been discussed in Chapter IV, and *nitrates* and *nitrituria* have been mentioned in Chapter XIV. The excretion of *ammonia* has been considered in Chapter IX. In

#### Daily Output of Salts in Health

		Gm. per diem
Chlorides	as NaCl	10 to 15
	as Cl	6 to 9
Sulphur (as SO <sub>2</sub> )	Inorganic sulphate	1.4 to 3.3
	Ethereal "	0.1 to 0.25
	Total "	1.5 to 3.5
	Neutral sulphur	0.15 to 0.4
	Total	1.6 to 3.6
Phosphorus (as P <sub>2</sub> O <sub>5</sub> )	Phosphates	1 to 5
	Organic phosphates	about 0.05
Carbonates	(fresh urine)	trace*
Nitrates	—	about 0.5

\* Ammonium carbonate is rapidly formed by the bacterial decomposition of urea.

#### Sulphur Partition of Normal Urine †

	The average Man on the average Mixed Diet.		An Individual on a Liberal Diet (Folin)		The same Individual on a Protein-deficient Diet (Folin)	
	Gm. per 24 hours	Sulphur as per cent. of total S	Gm. per 24 hours	Sulphur as per cent. of total S	Gm. per 24 hours	Sulphur as per cent. of total S
Inorganic SO <sub>2</sub>	2.92	88.2	3.27	90.0	0.46	60.5
Ethereal SO <sub>2</sub>	0.22	6.6	0.10	5.2	0.10	13.2
Neutral sulphur as SO <sub>2</sub>	0.17	5.2	0.18	4.8	0.20	26.3
Total SO <sub>2</sub>	3.3	100.0	3.64	100.0	0.76	100.0

† From Cole's *Practical Physiological Chemistry* (partly after Folin)

clinical work little or nothing of value is to be obtained from estimations of the output of *sodium*, *potassium*, *calcium* or *magnesium*, except in relation to metabolism experiments, for amounts excreted in health see table on p 294

Sulphur is excreted in three forms, inorganic sulphate, ethereal sulphate, and "neutral sulphur" (sulphides etc). Since the differential estimation of the three fractions is rarely of clinical value, the reader is referred elsewhere for the technique. In cystinuria (see Chapter IV), the cystine is included in the neutral sulphur fraction. Examples of detoxication by conjugation with sulphates to yield ethereal sulphates have been given in Chapters IV, VI XI and XIV. The simple inorganic sulphates are tested for by acidifying about 5 c c (1 in column in a test tube) of urine with concentrated hydrochloric acid, and adding an excess of 10 per cent barium chloride when a precipitate of barium sulphate is given by inorganic but not by ethereal sulphates. Normal urine always gives a precipitate. In poisoning with carbolic acid or with other substances which are conjugated with sulphates, the inorganic sulphates may be greatly reduced or absent, because most or all of the sulphate has been combined as ethereal sulphate. Apart from poisoning however, the test is of little or no use clinically.

The intake of all the above salts exerts so great an influence on their excretion, both in health and in disease, that only gross variations, such as can be demonstrated by qualitative tests, are of clinical significance. Quantitative estimations are practically limited to metabolism experiments. The upper table on p 289 shows the findings in health on an average mixed diet, and is given for reference. The lower table illustrates the sulphur partition of normal urine under different conditions of diet.

## CHAPTER XVI

### THE COLLECTION, PRESERVATION AND QUANTITATIVE ANALYSIS OF URINE

#### COLLECTION OF URINE

THE method of collecting urine for chemical examination necessarily varies with the particular analysis desired. Special methods have been noted under individual tests, *eg*, hourly collections in the urea concentration test (Chapter V).

For most quantitative analyses a sample of the twenty four hours' urine must be provided, and the total volume excreted must be measured. At a given hour (say 6 a.m.) the patient is instructed to empty his bladder completely and that sample is discarded. All subsequent specimens, including the one passed at the same given time (6 a.m.) twenty four hours later, are collected in a large receiver. The patient is told to micturate before defaecating, during the period of collection, so that no urine is lost. At the end of the period the bladder must be emptied completely. The volume excreted is measured in c.c. or in oz., and after thoroughly mixing the whole collection, a sample (200 c.c. or more) is taken, properly labelled, and sent to the laboratory with a note of the twenty four hours' volume. In certain circumstances the complete collection is forwarded to the laboratory.

This procedure is necessary in order that the day's output of a particular ingredient may be determined. The analyst actually estimates the concentration (*eg*, the number of grammes in 100 c.c. of urine) of the particular substance, and then, knowing the volume of urine in twenty-four hours, calculates the excretion of the substance per diem.

It must be remembered that, owing to the action of organisms, certain ingredients may be decomposed in the course of so long a period as twenty four hours, wherefore preservatives (see next section) have often to be added. Urea in particular is readily changed into ammonium carbonate by bacteria, so that preservatives should be added whenever urea or ammonia has to be determined (*cf* note on ammonia coefficient in Chapter IX). Glucose may be fermented by certain bacteria or by yeasts, so that for accurate work preservatives should be added when the day's excretion of glucose is to be estimated. For routine sugar analyses such preservation is generally unnecessary if the analyses are made within a few hours of the completion of the collection.

For routine qualitative tests the night's urine is usually collected. This is not only convenient for the nursing staff, but also desirable in that the night's specimen is commonly more concentrated than

samples collected during the daytime, owing to the absence of fluid intake during sleep. The night's urine is also less affected by diet, but in respect to glucose this is often a disadvantage. Glycosuria may occur after meals, but not after a night's "fast". When investigating cases of slight glycosuria it is important to remember this, and it is often valuable to collect the urine from meal to meal. In certain cases of intermittent proteinuria the night's urine may be free from protein, whilst the sample obtained a few hours after rising may contain an abundance (*cf* Chapter III).

Sometimes a comparison between the day and night urine is useful, *e.g.*, to demonstrate "fixation of the specific gravity" in chronic interstitial nephritis. In this disease the concentrating power of the kidneys may fail to such an extent that a urine of approximately uniform composition and specific gravity is excreted both by day and by night.

For most chemical tests it is not necessary to collect the urine with aseptic precautions. All vessels should be chemically clean and dry. For examination of urinary deposits fresh specimens should be provided, being more satisfactory than samples of the twenty four hours' urine.

### PRESERVATION OF URINE

As a routine method of retarding the multiplication of organisms, storage in the ice chest or cold room is probably the most generally useful, but it is important to remember that the growth of organisms is often only retarded and not completely inhibited. The combination of refrigeration and a chemical preservative is an improvement.

Toluene (1 c.c. per 100 c.c. of urine) is commonly employed as a preservative but is of very limited value. The urine after its addition must be well shaken. Toluene is inflammable. It leaves a greasy coating on all the glass surfaces with which it comes into contact. It is therefore likely to introduce errors into measurements with pipettes etc. If the urine originally contains many organisms, toluene often fails to stop their multiplication. The writer found it unsatisfactory, for instance, in preserving the urine during the twenty four hours' collection prior to estimations of the ammonia coefficient in cases of toxæmia of pregnancy.

Chloroform (sufficient to saturate the urine) has the advantage over toluene that it settles to the bottom instead of rising to the top. Its disadvantages are that it fairly readily volatilises, and that it reduces the cupric solutions used for testing for sugar. This latter difficulty is easily overcome by first boiling the urine to drive off the chloroform.

Thymol (sufficient of the crystals or of the powder to saturate the urine) is of some value as a preservative for short periods (one or more days, varying with the conditions), but there are several objections to its use. It lowers the surface tension and so may upset Hay's test (see Chapter XII). It modifies the indigo blue colour in oxidation tests for indican (see Chapter XIV), and it may interfere with other chemical reactions for certain urinary ingredients.

**Formalin** (added to the extent of 5 to 10 per cent formaldehyde, commercial formalin contains about 40 per cent formaldehyde) is useful for certain purposes, *e.g.*, the preservation of urinary deposits after these have been separated by centrifugation, but it reduces the cupric solutions employed in testing for sugar. Its use necessitates dilution, the magnitude of which must be measured in determining true percentage compositions, but which is immaterial in determining the day's excretion of a given substance, since the lowered concentration of the given substance is balanced by the increased volume of fluid recorded as the twenty four hours' measurement.

A saturated solution of perchloride of mercury is valuable for preserving urinary deposits after they have been separated by centrifuging, and washed in the centrifuge tube with physiological saline. The addition of the same salt to urine (*e.g.*, to yield a concentration of 0.05 gm. per 100 c.c. of urine) has been recommended as a general preservative, but is not entirely satisfactory.

The special use of sulphurous acid to prevent the oxidation of melanogen (1 c.c. of saturated  $\text{SO}_2$  solution per 100 c.c. of urine) and of homogentisic acid in alkaptonuria (5 c.c. of saturated  $\text{SO}_2$  solution per 100 c.c. of urine) have been mentioned in Chapters XI and VI.

For estimations of urea, ammonia, and total nitrogen, and indeed for most purposes, the twenty four hours' urine may be successfully preserved by making it strongly acid (*pH* under 5.0). Brandt and Stokstad recommend the addition of about 1 c.c. of concentrated hydrochloric acid to every 100 c.c. of urine. Addis and Watanabe employed 60 c.c. (2 oz.) of approximately 2N sulphuric acid (56 c.c. of concentrated  $\text{H}_2\text{SO}_4$  in 1,000 c.c. of water) for the whole twenty-four hours' collection. The 60 c.c. of acid are placed in the large receiver, and the samples of urine added immediately after they have been passed, mixing the contents of the jar after each addition. Acidification is the most satisfactory method of preserving urine for chemical analysis, except on those occasions when the addition of acid is objectionable. It is often necessary to neutralise the added acid with alkali before making the analysis. Cellular deposits are destroyed, and uric acid is precipitated.

### QUANTITATIVE ANALYSIS

The techniques of the estimations most commonly requested in clinical work have already been described (protein in Chapter III, urea in Chapter V, "sugar" in Chapter VI, and diastase in Chapter XIII). Recently *pH* determinations, in controlling the treatment of urinary infections by ketogenic diet, or by mandelic acid plus ammonium chloride, or by ammonium mandelate, and the estimation of vitamin C (ascorbic acid) have been introduced into clinical work, and are described at the end of this chapter. For other quantitative methods the reader is referred to the standard works on chemical physiology, etc.

*Normal Urine : Twenty-four Hours' Output on a Mixed Diet \**

Acetone bodies (total, as acetone)	20 to 50 mgm	
Acetone plus aceto acetic acid (as acetone)	3 to 15 mgm	
$\beta$ hydroxybutyric acid (as acetone)	20 to 30 mgm	
Amino acid nitrogen	0.4 to 1.0 gm	
Ammonia	0.5 to 1.0 gm	
Calcium, as Ca	0.01 to 0.3 gm	
Creatine	Nil or traces	Infants and children 10 to 15 mgm
Creatinine	1.0 to 1.5 gm	7 to 11 mgm. per kgm. of body weight.
Diastase	8,000 to 30,000 units	Index of 6.7 to 33.3 Wohlgemuth units.
Freezing point	-1.0 to -2.5° C	
Glucose	About 0.5 gm	
Hippuric acid	About 0.7 gm	
Indican	0 to 32 mgm	Usually 5 to 20 mgm
Iron, as Fe	1 to 10 mgm	
Magnesium, as Mg	0.05 to 0.2 gm	
Organic acids	About 6 cc of 0.1 N per kgm	<i>J. Biol Chem</i> , 1920, 41, 567.
Oxalic acid	15 to 20 mgm	
Potassium, as K	2 to 4 gm	
Protein	Nil, or traces of mucus only	
Purine bases	16 to 60 mgm	
Reaction	pH 4.8 to 7.4	Mean pH 6.0
Sodium, as Na	3 to 6 gm	
Specific Gravity	1.010 to 1.025.	
"Sugar" (total reducing substances)	1.0 to 1.5 gm	
Total nitrogen	10 to 17 gm.	See also p 295
Total solids	55 to 72 gm	
Urea	20 to 35 gm	See also p 295.
Uric acid	0.1 to 2.0 gm	Average 0.7 gm
Volatile fatty acids	8 to 50 mgm.	
Volume	1,000 to 1,800 c.c.	Average 1,500 c.c. or about 1 cc per minute

As indicated in Chapter XV, the day's output of most of the urinary ingredients depends largely, and often almost entirely, on the intake of those ingredients or their precursors. For this reason and owing to the practical difficulties of accurately collecting the twenty-four hours' urine, except in special metabolism wards with their fully-trained nursing staffs, the clinician's attention of recent years has been more and more focussed on blood analyses. Nowadays, therefore, excluding estimations of the substances mentioned in the preceding paragraph, urine analysis is seldom requested. The table on p 294 and the nitrogen partition of normal urine, however, are given for reference. The main inorganic constituents have been discussed in Chapter XV.

*Nitrogen Partition of Normal Urine \**

	The average Man on the average Mixed Diet			An Individual on a Liberal Diet. (Folin)			The same Individual on a Protein-deficient Diet (Folin)		
	Gm per 24 hours.	Nitrogen per 24 hours.	Nitrogen as per cent. of Total Nitrogen.	Gm. per 24 hours.	Nitrogen per 24 hours.	Nitrogen as per cent. of Total Nitrogen.	Gm per 24 Hours.	Nitrogen per 24 hours.	Nitrogen as per cent. of Total Nitrogen.
Urea . . .	30.0	14.0	87.5	31.6	14.7	87.5	4.72	2.2	61.7
Ammonia . . .	0.8	0.5	3.1	0.6	0.49	3.0	0.51	0.42	11.3
Creatinine . . .	1.55	0.57	3.6	1.55	0.53	3.6	1.61	0.60	17.2
Uric acid . . .	0.7	0.23	1.4	0.54	0.18	1.1	0.27	0.09	2.5
Undetermined . . .	—	0.7	4.4	—	0.85	4.8	—	0.27	7.3
Total—N. . .		16.0	100.0		16.8	100.0		3.6	100.0

\* From Cole's *Practical Physiological Chemistry* (partly after Folin).

*Note* The approximate percentage composition of a sample of the 24 hours urine of a healthy man on a mixed diet is urea 15 to 20, ammonia about 0.04, creatinine 0.07 to 0.10, and uric acid about 0.05 per cent.

### DETERMINATION OF URINARY pH

The simplest method is to employ the Lovibond comparator<sup>1</sup> (Fig. 52), with a disc of nine glasses for each indicator in steps of 0.2 pH. Special comparator tubes of identical bore are supplied.

Measure 10 c.c. of urine into the right-hand comparator tube,<sup>\*</sup> add 0.5 c.c. of indicator solution and mix. Place about 10 c.c. of untreated urine in the left-hand tube to compensate for the urinary colour. Rotate the disc until the colours match and read the result.

<sup>1</sup> Supplied with the necessary discs, indicator solutions and tubes by Messrs. The Tintometer Ltd., Milford, Salisbury, or by Messrs. The British Drug Houses Ltd., London, N.1.



Four indicators cover the range of  $pH$  in urine, viz —

Indicator	$pH$ Range	Colour Changes Acid to Less Acid or Alkaline
BDH ' 4460	4.4 to 6.0	Red orange yellow green
Brom cresol purple	5.2 to 6.8	Yellow to purple
Brom thymol blue	6.0 to 7.6	Yellow to blue
Phenol red	6.8 to 8.4	Yellow to red

It is possible to dispense with the brom thymol blue indicator and the corresponding disc

The concentration of indicator solution (0.04 per cent) supplied by Messrs The British Drug Houses Ltd for the test is the same as that used in preparing the buffer plus indicator solutions of definite  $pH$  against which the coloured glasses originally were matched

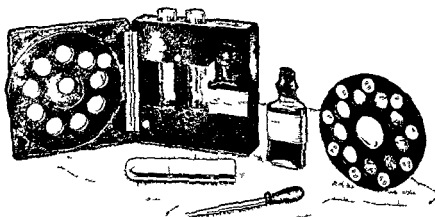


FIG 52 The Lovibond comparator, opened to show contents, and a disc with nine coloured glasses For external view see Fig 7 p 36

Naturally the urine must be fresh and certainly not more than one hour old when the test is made. If not clear it should be centrifuged or filtered, a slight residual opalescence due to bacteria makes the "unknown" less bright than the "standard," but with a little practice there is no difficulty in deciding the  $pH$  by comparison of the tint or hue of the "unknown" with that of the standard glasses. It is easy to see when the colour of the unknown is about half way between two consecutive glasses, so that readings can be made approximately to the nearest 0.1  $pH$ .

The method is based on the usual colorimetric principle for urinary  $pH$  determinations, the urine plus indicator has a colourless glass in front of it instead of a tube of distilled water, the  $pH$  buffer-standard solution plus indicator is replaced by the coloured glass, and is backed by untreated urine.

The great advantage of this technique is its simplicity, and the fact that the standard glasses are permanent.

### Note on Control of Urinary pH in Urinary Infections

Fuller concluded that the principal factor inhibiting the growth of bacteria in treatment by ketogenic diet was  $\beta$  hydroxybutyric acid, and that the activity of this substance increased in proportion to the acidity of the urine. Rosenheim introduced mandelic acid, phenyl hydroxy acetic acid,  $C_6H_5 \cdot CH(OH) \cdot COOH$  as a substitute, and thereby avoided the rigid diets required in the first method. Twelve grammes daily, divided into four doses were recommended —

Mandelic acid	. 3 gm
Sodium bicarbonate	. 1.6 gm.
Lemon to flavour	
Water	to 30 c c

The fluid intake daily was limited to 2 pints. In both methods it is essential to keep the urinary reaction markedly acid *e.g.*, pH between 4.8 and 5.3 for which purpose acidifying salts are employed. Rosenheim used 1 gm. of ammonium chloride in a cruet four times daily, increasing the daily dose by steps up to 8 gm. if the urine did not become sufficiently acid. He emphasised the possible danger of acidifying salts and pointed out that the occurrence of casts or of proteinuria indicated caution and that ammonium chloride should not be given in the presence of renal failure. Acidosis of a serious grade is easily produced by acidifying salts (*cf* Chapter IX), and it is probably this and not the neutralised mandelic acid which has been responsible occasionally for clinical signs which may be alarming and serious if treatment is continued. The risks of acidosis can easily be measured by determinations of the alkali reserve (Chapter IX).

Ammonium mandelate syrup<sup>1</sup> containing the equivalent of 3 gm. of mandelic acid four times in the day, has largely replaced sodium mandelate plus ammonium chloride. The ammonium mandelate alone often makes the urine sufficiently acid, but in some cases ammonium chloride has to be given as well to keep the reaction below pH 5.3. Infections with *B. proteus* may be particularly resistant to treatment, owing to the difficulty of making the reaction sufficiently acid, since this organism forms ammonia from urea, it may be impossible to make the urine acid at all.

### ESTIMATION OF VITAMIN C (ASCORBIC ACID) IN URINE

Using Tillman's reduction indicator, 2.6 dichlorophenolindo phenol. Harris and Ray introduced an easy technique which is of clinical value in the diagnosis of scurvy, the detection of vitamin C subnutrition, or in demonstrating that the diet is not deficient in ascorbic acid. This is the first vitamin for the estimation of which the physician has been provided with a simple method.

<sup>1</sup> Ammonium mandelate is hygroscopic and very soluble in water, 3.34 gm. is equivalent to 3 gm. of mandelic acid.

### Preparation and Standardisation of the Dye Solution

Treat 0.025 gm of 2,6-dichlorophenolindophenol with about 10 c.c. of boiling distilled water. Decant the supernatant fluid into a 50 c.c. volumetric flask. Repeat the extraction with similar portions of boiling water until all the dye is dissolved, cool and make up to 50 c.c., 1 c.c. = 0.5 mgm of dye.

Dissolve 0.025 gm of "pure" ascorbic acid in water to 50 c.c. and check its purity by titration against 0.01 N iodine solution. Thus, measure 5 c.c. of the ascorbic acid solution into a boiling tube or beaker, and run in the 0.01 N iodine solution from a microburette, using starch as indicator.

$C_6H_8O_6 \equiv I_2$  wherefore 176 mgm of ascorbic acid should require 200 c.c. of 0.01 N iodine. Therefore 5 c.c. of 0.05 per cent ascorbic acid, or 2.5 mgm, should need  $\frac{200}{176} \times 2.5$  or 2.84 c.c. of 0.01 N iodine solution.

Having thus checked the purity of the ascorbic acid solution, use it to standardise the dye solution. To 1 c.c. of the 0.05 per cent 2,6-dichlorophenolindophenol add 3 drops (0.1 c.c.) of glacial acetic acid and run in the 0.05 per cent ascorbic acid from a microburette, until all the red colour is discharged. It should need about 0.5 c.c., i.e., 0.5 mgm of dye should correspond to about 0.25 mgm of ascorbic acid. Calculate what is the exact ascorbic acid equivalent of the dye and note it for future use.

The solid dye generally remains unaltered for at least two months, it is wise to check it against ascorbic acid at intervals of one month. The 0.05 per cent solution of dye keeps for about a week, but for safety should be checked as above described against freshly prepared ascorbic acid solution every three days.

Solid ascorbic acid appears to be fairly stable, but each new batch should be checked against 0.01 N iodine as above described. Solutions of ascorbic acid oxidise rapidly in air, and should be used as fresh as possible, *e.g.*, within one hour of preparation.

### Technique for Fresh Urine

The urine must be titrated "at once," for practical purposes within half an hour of its being passed. Acidification permits of a slight delay—see next section.

Measure exactly 0.5 c.c. of the 0.05 per cent dye solution into a boiling tube, add 1.0 c.c. of glacial acetic acid, and run in the urine from a 25 c.c. burette<sup>1</sup> quickly until the red colour just disappears. The concentration of acetic acid finally should be about 10 per cent, if therefore the volume of urine required is not close to 10 c.c. repeat the test with the appropriate amount of the acid.

The titration must be completed within two minutes, if it is prolonged, substances other than ascorbic acid will also cause some reduction of the dye, also if it is performed extremely rapidly, *e.g.*,

<sup>1</sup> Use a microburette of 5 c.c. or 2 c.c. capacity for urines rich in ascorbic acid as for instance after the administration of large doses of the vitamin (p. 300).

within thirty seconds, too much urine will be added—reduction of the dye by urinary ascorbic acid is not instantaneous.

With a little practice the end point is quite satisfactory, except for urines very low in vitamin C, the dye then becomes so diluted that it is difficult to decide whether dilution or reduction is responsible for the disappearance of the colour, and comparison with urine plus  $\frac{1}{10}$  volume of acetic acid but no dye is then useful.

**Example of Calculation** 0.5 mgm of the solid dye in use was equivalent to 0.25 mgm of ascorbic acid, 0.5 c.c. of 0.05 per cent dye solution was employed for the test, i.e., 0.25 mgm of the dye which equals 0.125 mgm of ascorbic acid. In the titration 10 c.c. of urine were required. Therefore 10 c.c. of urine contained 0.125 mgm of ascorbic acid. Therefore 100 c.c. of urine contained 1.25 mgm. The total volume of the sample of urine was 440 c.c. Therefore the whole specimen contained  $4.4 \times 1.25$  or 5.5 mgm of ascorbic acid.

### Preservation of Urine by Acidification

It is best to use fresh urine, but is not always convenient, e.g., for samples passed in the night. Harris and Ray recommended the addition of 1 volume of glacial acetic acid to 10 volumes of urine as preservative, but in spite of this some oxidation of the ascorbic acid may occur even in four hours. Acidification, however, definitely retards the oxidation, which moreover can be reversed by treatment with  $H_2S$  up to a period of about eighteen hours. Without acidification a significant fraction becomes irreversibly oxidised in a few hours, and there may be a total loss in twenty four hours.

### Regeneration of Ascorbic Acid by $H_2S$

Measure the volume in cubic centimetres of each sample of urine immediately after it is passed, and add  $\frac{1}{10}$  volume of glacial acetic acid, or provide bottles, each containing 10 c.c. of glacial acetic acid to which the specimens are added at once. On receipt in the laboratory measure the volumes and add sufficient acid to make its final concentration 10 per cent.

As soon as convenient, and certainly within eighteen hours, saturate each sample with  $H_2S$  by passing the gas for about ten minutes. Remove the  $H_2S$  completely by means of a vigorous current of  $CO_2$  or of nitrogen for at least thirty minutes. Test a small portion by adding a few drops of a concentrated solution of lead acetate, compare with the same amount of another sample of the same urine (or of normal urine) which has not been treated with  $H_2S$  at all, but to which a similar amount of lead acetate has been added. There is a precipitate which is white if the removal of  $H_2S$  has been complete, whereas any browning or blackening, due to  $PbS$ , indicates incomplete removal. Finally use the rest of the urine, after removal of  $H_2S$ , for the titration just as described above, except that no further acetic acid is added.

**Example of Calculation** The volume of the sample of urine was 300 c.c., to which were added 30 c.c. of glacial acetic acid, making a total of 330 c.c. Titration of 0.5 c.c. of dye solution,

which corresponded to 0.125 mgm of ascorbic acid (see p. 299), required 15 c.c. of treated urine. Therefore the total 330 c.c. contained  $\frac{330}{15} \times 0.125$  or 2.75 mgm of ascorbic acid.

### Testing the Saturation of the Subject with Ascorbic Acid

Healthy individuals show wide variations in the excretion of ascorbic acid according to their diets, but commonly excrete from 10 to 60 mgm each day. Harris *et al.* regard figures below 13 mgm per diem as suggestive of vitamin C subnutrition. Cases of frank scurvy excrete from 0 to about 20 mgm daily. An estimation of the twenty-four hours' output therefore may be difficult to interpret, figures of 30 mgm and over exclude scurvy and vitamin C subnutrition, but the significance of lesser amounts can only be decided after studying the response to the administration of test doses of ascorbic acid.

The general trend of results after test doses is clear. The individual in good nutrition responds at once by an increased output of ascorbic acid in the urine, his tissues are more or less saturated with vitamin C. The sub-scorbutic state is revealed by a poor immediate additional excretion, the tissues are not saturated and for a few days take up most of the ascorbic acid administered. In frank scurvy it is necessary to continue the large doses for several days to obtain any increase of excretion of ascorbic acid, it takes relatively a long time to saturate his tissues.

The therapeutic test, or test of ascorbic acid saturation, is, however, still in the experimental stage, and it is impossible to say yet what is the best way of applying it. Harris *et al.* have used mostly a single test dose of 600 mgm, or 10 mgm of ascorbic acid per kilogramme of body weight. Archer and Graham noted the total of ascorbic acid which had to be given before the subject excreted 75 per cent. of his day's intake of 1,000 or 400 mgm. For details the reader is referred to the papers of the authors cited. There is no difficulty in recognising cases which had previously received diets grossly deficient in, or amply supplied with vitamin C, but it is likely that the intermediate states of vitamin C nutrition will grade into one another. None the less the therapeutic test undoubtedly assists in judging the state of vitamin C nutrition, and in assessing the significance in the preliminary test of a twenty-four hours' output of less than 30 mgm of ascorbic acid.

**Notes.** Medes has published a colorimetric method for determining ascorbic acid in urine with phosphotungstic acid, which she claims is more accurate for urines rich in the substance. Technically (see particularly her second paper) it is more complicated than the dye method and is unlikely to be preferred for clinical work.

With urines low in vitamin C there is little doubt that the titration method includes small quantities of reducing substances other than ascorbic acid, and several modifications have been introduced to overcome this. At present there is no general

agreement as to what is the best technique, and the writer feels that the simplest method as above described is so far the best for clinical work.

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## CHAPTER XVII

### THE COLLECTION AND PRESERVATION OF BLOOD

#### COLLECTION

THE site from which the blood is collected varies with the amount required and the nature of the analysis desired

Capillary blood is obtained by pricking the finger, puncturing the lobe of the ear, or stabbing the heel. In the case of the finger a piece of rubber tubing is wound round from above down towards the tip of the finger, so as to make the end of the digit full of blood. Or the operator, with his own thumb and second and third digits

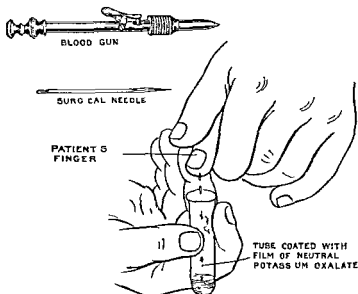


FIG 53 Collection of blood from finger

may compress the patient's finger successively from base to apex (Fig 53). The finger must be warm to ensure a good flow of blood. If it is not, it should be warmed, either by massage, or by immersing the hand in hot water (and then drying it). Swinging the arm round and round and hanging it down also helps. The prick is made with a sterile large surgical straight triangular needle, or 'blood gun' (Fig 53). The stab may be made close to the base of the nail, but it is generally more convenient to use the extreme end of the digit (Fig 53). Pressure on the nail will increase the amount of blood ejected. Both for the patient's comfort, and to reduce the risk of clotting, excessive squeezing and undue constriction should be avoided. Having squeezed out as much as will flow readily, the

constriction is released, the finger allowed to fill again with blood, and the process repeated. One cubic centimetre, and often more, may easily be secured.

In the case of the ear, it is best to punch out a small piece of skin with a glass pricker. The latter is made by drawing out slowly a piece of glass tubing in the blowpipe (Fig 54, *a*). The capillary portion is broken in the middle. Each of the resulting two pieces may be made into a pricker as follows. The capillary, at its junction with the main tube, is placed in the lower (cooler) part of a bunsen flame, and when just red hot is drawn out slowly (Fig 54, *b*). When cool the major portion of the capillary is snapped off, leaving a stump of capillary attached to the original tube. This stump is cut short till its cross section is of the desired size, by drawing it sharply across the detached capillary, the detached capillary thus acting as a "cutting" edge (Fig 54, *c*). The end of the pricker is

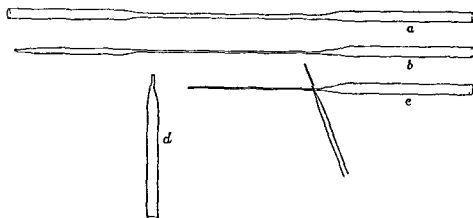


FIG 54 Stages in the preparation of a sterile glass pricker

sterile, and must be left rough (Fig 54, *d*). The ear is made thoroughly warm by the patient, who is asked to rub it with the palm of his hand. The pricker is then "bored" into the most fleshy part of the lobe. When removed, a tiny plug of skin is often seen in the tip of the pricker. The shoulder on the pricker, if it is made as illustrated, prevents it being pushed in too far. The lobe is grasped between the first and second digits of the one hand and the third and fourth digits of the other hand, and the base of the "blob" of lobe is firmly but not ferociously constricted (Fig 55, *a*). By approximating the two hands (Fig 55, *b*) a large drop of blood is "milked" out. On releasing the grip the lobe fills rapidly with blood, when the process is repeated. The milking is not started, of course, till the initial spontaneous flow of blood is stopping. This method requires a little practice, but once mastered, is very useful. It is easy to collect 3 to 5 c.c. of blood in this way. The method is particularly helpful when running blood (0.1 or 0.2 c.c.) straight into a pipette for blood sugar estimations, etc. (See Fig 55, *a*). When taking samples at half hourly intervals in the sugar tolerance



test, one prick with the glass pricker will often suffice for the whole curve since a tiny piece of skin is punched out. At the end of each half hour, the ear is warmed the clot removed with the aid of a piece of moistened lint, the lobe dried, and the milking process started

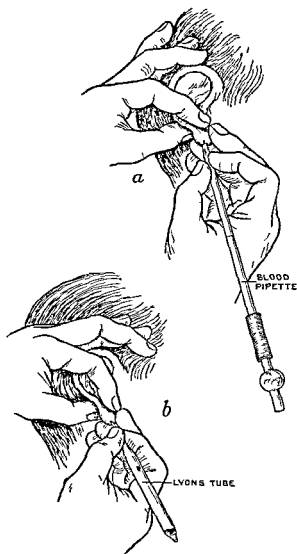


FIG 55 Collection of blood from ear a Directly into a pipette b Into a Lyons tube

again. The whole secret of success lies in the avoidance of pinching, which is not only painful to the patient but also rapidly causes the blood to clot. Contrary to what might be expected the pricker causes little if any, more pain than a needle. Some workers use a cataract knife, instead of a pricker, and make a small incision in the lobe.

It is often difficult to obtain blood from infants. The best way

to secure capillary blood is to grasp the ankle firmly so as to make the foot congested, and then to stab the heel with a stout surgical

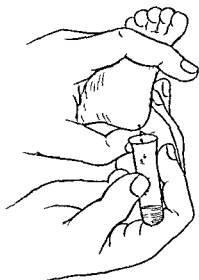


FIG 56 Collection of blood from infant's heel

needle The blood is then "milked out" by massaging from the toes down to the heel, gripping the whole foot in the hand (Fig 56)

Venous blood is obtained from one of the veins at the bend of the elbow, or from any cutaneous vein made prominent by suitable constriction (Fig 57) Franklin and McLachlin have shown that veins dilate in response to tapping, and suggest that this may be of

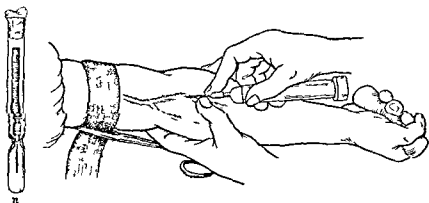


FIG 57 Obtaining blood from a superficial vein of the arm n Needle with rubber tubing sterilised in plugged tube

practical value in difficult cases The part is cleansed, *e.g.*, with alcohol and ether, or with acetone, the constriction is applied, and the needle is inserted in the vein, which is fixed with the thumb or forefinger (Fig 57) The blood is then collected Before withdrawing the needle, the constriction is released, so that a hæmatoma does not result on taking out the needle Immediately after withdrawing

the needle, a wad of wool and digital pressure is applied for two or three minutes. If the puncture is made in front of the elbow the arm should be kept extended. Flexing the elbow tends to impede the collateral circulation, and to pucker up the skin so that blood readily collects in the loose subcutaneous tissues. A piece of bicycle inner tube makes a most excellent and comfortable broad tourniquet. In cases of difficulty the whole arm may first be immersed in a warm bath till the veins are nicely distended. In adults, a needle with a rubber connection is often used for taking the sample (Fig 57, n). In children, and in any case in which difficulty is anticipated, it is generally best to use a syringe, so as to be able to apply suction. The syringe may with advantage be lubricated with liquid paraffin, except when "fats" are to be estimated. After use, the syringe and needle are washed out with tap water at once, and later with distilled water and acetone. The syringe then rapidly dries, after which it is lubricated again with liquid paraffin. The needle, re sharpened if necessary, may be placed in a small tube, projecting into the lumen of which are three protuberances. Each of these is made by heating to red heat in the blowpipe flame a *small* area of the wall of the tube, which is then pushed in with the point of a file. The shoulder of the needle rests on the protuberances, so that the point does not reach the bottom of the tube, which is plugged with cotton wool and sterilised in the hot air oven (Fig 57, n).

In young children a superficial vein in the neck is often selected, and the child is best placed on its side on a rigid table with the head

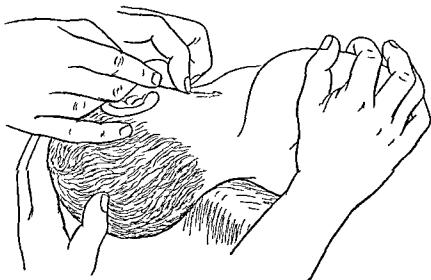


FIG 58 Collecting blood from a child's superficial neck vein

hanging over the edge, on which is placed a small pillow (Fig 58). A table is much more satisfactory than a bed.

In infants whose fontanelle is patent, sinus puncture is much the simplest and most satisfactory method in almost all cases. There

is not the least danger if one golden rule is observed, *viz*, *never bury the needle deeper than the length of its bevel*. The infant is placed on its back across the bed or on a table, and the head is securely held by an assistant, who places his hands firmly one over each ear. *The head must be held rigid, no movement being allowed*. The skin over the anterior fontanelle is painted well with iodine. The needle is inserted in the posterior angle of the fontanelle in the mid line, and as already stated is pushed just through the skin (Fig 59). If blood is not obtained the needle is not pushed in any farther, but is removed and reinserted slightly to the right or left. It is a help if the patient's head is just short of the edge of the bed or table, so that there is room for the operator to rest and steady his hand. It is a good plan to turn the bevel of the needle downwards, towards the bed, so that if the hand drops a little the bevel falls

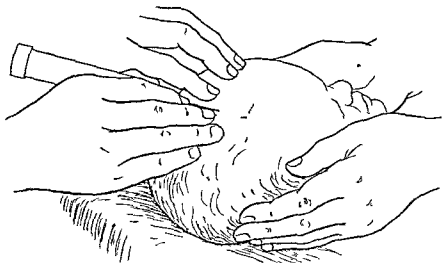


FIG 59 Collecting blood from an infant's longitudinal sinus

towards the scalp. If the bevel is turned uppermost and the operator's hand drops, there will often be a leakage of blood—so short is the distance between the surface of the skin and the lumen of the vein. With observance of the above directions cerebrospinal fluid will seldom, if ever, be drawn. Many thousands of punctures have been performed on out patients in children's hospitals without any accidents, the operation being no more serious than any other form of vein puncture. This is mentioned because the writer has not uncommonly met with marked aversion to the procedure. If cerebrospinal fluid should accidentally be tapped, pressure should be applied to the site of puncture, the infant should be placed upright and kept under observation till the flow has ceased—usually only a matter of a few minutes.

Arterial blood is obtained by puncture of the radial, brachial or femoral artery with the aid of a fine needle and syringe, and, usually, local anæsthesia. At present this method is used only occasionally in clinical work, but when it becomes generally recog-

nised that there is no appreciable risk attached in skilled hands, no doubt it will be more widely used, and it will enable much valuable information to be acquired. Arterial blood is particularly important in investigations involving blood gas analysis.

### Apparatus for Collecting Blood.

Capillary blood may be collected in a small test tube (Fig 53 and p 309) in a crucible, in a Wright's or Lyon's tube (Fig 55, *b*), or directly into a pipette (Fig 55, *a*).

When obtaining venous blood, an all glass syringe with solid plunger is best. A hollow plunger, however, is often satisfactory if lubricated with liquid paraffin. Obviously the syringe must not contain physiological saline or water if quantitative estimations are to be made on the blood, owing to the introduction of an unknown dilution. A "Behring venule" (obtainable from Messrs Bayer Products Ltd, London) is convenient for those who only occasionally perform vein punctures. Those who have the facilities of a chemical pathological laboratory, or for sharpening and sterilising their own needles, will find the apparatus previously described less expensive in the long run.

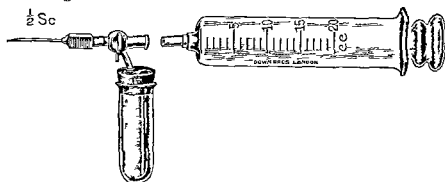


Fig 60 Syringe with Emery's two way tap for collection of blood without loss of gases and without admission of air

When blood is to be collected directly under paraffin to prevent loss of gases, the device illustrated in Fig 60 is useful. With the Emery two way tap open to air, the piston of the syringe is removed. With the tap closed, the requisite amount of neutral potassium oxalate powder is tipped into the barrel. Liquid paraffin is then poured down the inside of the barrel carefully to avoid inclusion of air bubbles. The piston is dipped in liquid paraffin and is then pushed into the barrel  $\frac{1}{2}$  in or so, the syringe is slowly inverted so that the nozzle is uppermost, allowing the paraffin to run slowly down on to the top of the piston. The tap is opened and all air is displaced by slowly pushing up the piston. The tap is then closed. A sterile needle is fitted and the vein (or artery) is punctured. The tap is turned to connect with the side tube and blood is allowed to flow through into the little glass receiver, so as to displace all air from the needle. Incidentally, this device enables the operator to see whether blood is flowing freely. The tap is then turned to

connect with the syringe, and the blood is collected under the paraffin without admission of air, or loss of gases from the blood. The tap is closed, the needle withdrawn, and the syringe is rotated to ensure solution of the oxalate.

Van Slyke and his colleagues, in accurate work on blood gas analyses and electrolyte determinations, find it best to collect the blood over mercury. The reader is referred to Peters and Van Slyke, *Quantitative Clinical Chemistry*, Vol. II, for a discussion of this and other methods of anaerobic drawing and preservation of blood.

## PRESERVATION FOR ANALYSIS

**Prevention of Clotting** Neutral potassium oxalate or sodium citrate is generally used. In special work in which anti coagulants are objectionable, clotting may be retarded for a few minutes by lubricating the needle and syringe with sterile liquid paraffin, and by lining the centrifuge tube with paraffin wax. Heparin and heparin are rarely used in clinical work. A final solution of 0.2 to 0.3 gm. of neutral potassium oxalate,  $(\text{COOK})_2 \cdot 4\text{H}_2\text{O}$ , per 100 c.c. of blood is the concentration at which to aim. Clotting may not be prevented by concentrations of less than 0.15 per cent, and amounts exceeding 0.5 per cent are apt to interfere with the accuracy of certain analyses. It is not necessary to weigh out the oxalate each time. In the first instance, say, 0.025 gm. of the dry finely powdered oxalate is accurately weighed into a tube in which 10 c.c. of blood are to be collected, and thereafter a pinch of approximately the same volume as the 0.025 gm. is taken. Of course, proportionately smaller or larger amounts may be used, depending on the volumes of blood to be collected. It is often advisable to make the oxalate very finely divided, and to coat the walls of the vessel with it. If this precaution is not observed, for instance, when collecting capillary blood in a tube, clotting is almost certain to occur, because the blood starts to congeal as it runs down the side, and because powdered oxalate is relatively coarse and takes a fair time to dissolve in blood. The following is a convenient method for lining a tube with finely divided oxalate —

The tubes should be about 55 mm. long and 12 mm. in diameter (external measurements). Fill one tube with a 5 per cent solution of  $(\text{COOK})_2 \cdot 4\text{H}_2\text{O}$  and tip the contents into a second. The walls of the first tube are thus moistened with about 0.1 c.c. of the solution. Place the tube on a hollow wire gauze and rotate it over a flame until just dry. Avoid overheating which converts the oxalate into carbonate. The thin film of about 5 mgm. of oxalate will prevent coagulation of the blood in amounts up to about 2 c.c.

In certain circumstances it is more convenient to add a saturated solution of oxalate to the blood immediately after withdrawal. One or more drops of known volume are added to the blood. Thus, 1 drop from a pipette delivering 15 drops to the cubic centimetre added to 5 c.c. of blood gives approximately a 0.3 per cent solution. It is convenient to collect the blood in (centrifuge) tubes marked

at 5, 10, and 15 c c, and to add the requisite number of drops of saturated oxalate solution. This method introduces a slight dilution error of the order of 1 to 2 per cent, but this may be neglected or allowed for. The advantage of the saturated solution as opposed to solid oxalate powder is that the former is less likely to cause hæmolysis.

In the case of sodium citrate,  $C_6H_5O_7Na_3 \cdot 5\frac{1}{2}H_2O$ , a final concentration of 0.5 to 0.75 per cent is aimed at. Less than 0.3 per cent may not prevent clotting. One drop ( $\frac{1}{15}$  c c) of a saturated solution of citrate to 5 c c of blood gives approximately a 0.6 per cent solution. This is even less likely to cause hæmolysis than a saturated solution of oxalate. When estimating calcium, oxalate must not be used, because the calcium would be precipitated as calcium oxalate. Citrate is safe because a soluble, though non ionised calcium compound is formed.

**Prevention of Changes in Shed Blood (Glycolysis, etc.)** Often the blood analysis cannot be carried out immediately after withdrawal. In that case changes in the shed blood must be prevented or retarded. This holds particularly in sugar estimations. Glycolysis may start in shed blood in as short a period as fifteen minutes under certain conditions, but as a rule oxalated blood may safely be kept corked at room temperature for an hour or so. Many reagents have been suggested to prevent clotting plus glycolysis. The two following are probably the best (*cf* Evans, John, Sander).

- |                     |        |                        |
|---------------------|--------|------------------------|
| (1) Sodium fluoride | 1.0 gm | } per 100 c c of blood |
| Thymol              | 0.1 gm |                        |

The two substances are finely powdered and mixed thoroughly together in the above proportion (10 to 1). Of the mixed powder 0.1 gm is convenient for 10 c c of blood. (Some workers recommend smaller amounts even down to one fifth of the above i.e. 0.2 per cent *cf* inorganic phosphate p. 366.) This powder will prevent glycolysis for one to two weeks and will preserve the blood for analysis of sugar, uric acid, creatinine and non protein nitrogen for several days.

- |                           |        |                        |
|---------------------------|--------|------------------------|
| (2) Sodium fluoride       | 0.1 gm | } per 100 c c of blood |
| Neutral potassium oxalate | 0.3 gm |                        |

This will prevent clotting and glycolysis for two or three days.

These preservatives are particularly useful when samples have to be sent through the post, but they must not be used if urea is to be determined by a urease method, because fluoride is an 'enzyme poison'. In practice a tube containing fluoride thymol, or fluoride oxalate, is provided when sugar is to be determined and a plain oxalate tube is sent when urea is to be estimated, the urea in oxalated blood does not alter in twenty four hours.

**Prevention of Loss of Gases** Before making gas analyses, and also in the estimation of plasma chlorides, it is necessary to prevent loss of gases from the blood. At the moment of withdrawal the blood is in equilibrium with the gaseous content of the surrounding tissues. Arterial or capillary blood has approximately the same tension of  $CO_2$  as alveolar air (normally about 5 per cent or 40 mm of  $CO_2$ ). Venous blood contains more  $CO_2$ . When exposed to atmospheric air,  $CO_2$  is rapidly given off, because the ordinary air

only contains some 0.03 per cent of  $\text{CO}_2$ . For this reason shed blood plasma would tend to become more alkaline, but this is compensated for, in part at any rate, by migration of ions (and particularly of chlorine ions) from the corpuscles into the plasma. It follows therefore that the plasma of shed blood exposed to the ordinary air becomes richer in chlorides than the original "true plasma" circulating in the vein. To prevent this "chloride shift" the blood must be collected without loss of gases (see p. 179). In most clinical work, the blood may be drawn into a centrifuge tube containing oxalate, after mixing it is covered with a layer of paraffin, centrifuged as soon as possible, and the plasma separated.

### THE CHOICE OF WHOLE-BLOOD, PLASMA OR SERUM

For most analyses whole blood (suitably oxalated or citrated) is preferred. Plasma is generally used rather than serum in chemical work because plasma can be separated more quickly. Whole blood is the more economical, but plasma analyses represent more accurately the chemical composition of the actual fluid supplying the needs of the tissues. This holds in the case of certain constituents (chlorides, ionised hydrogen, etc.) only when "true plasma" is employed (see above). To obtain 5 c.c. of plasma it is usually necessary to draw 10 c.c. of blood or slightly more. Serum, roughly speaking is plasma less fibrin.

In some cases (e.g., urea) the constituent is approximately evenly distributed between corpuscles and plasma, so that it is immaterial whether whole blood, plasma or serum is used. Urea is accordingly generally estimated in whole blood. Sugar likewise is almost always determined in whole blood. When examining the blood for abnormal pigments, plasma or serum is used, and hæmolysis must be avoided (see "Icterus Index," Chapter XII). In a few instances (e.g., sulphæmoglobin (Chapter XVIII)) the abnormal pigment is intracorporeal, so the sediment of corpuscles obtained by centrifuging, or whole blood, is examined. Serum or citrated plasma, but not oxalated plasma, for reasons already given, is required for calcium determinations.

From the table on pp. 333 to 336 it will be seen that certain substances are not evenly distributed between corpuscles and plasma. It is therefore essential to state in the report of the analysis whether whole blood, plasma or serum has been employed. In the case of blood sugar findings it is important to note whether capillary or venous blood has been used (cf. Chapter VII).

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## CHAPTER XVIII

### BLOOD : QUALITATIVE EXAMINATION

**Books** The information collected in this chapter is scattered through the books on Chemical Physiology, Clinical Pathology and so on. One book gives a comprehensive account of one portion of the subject, another book treats a second portion well, but the writer has been unable to find any one exhaustive treatise. Perhaps the fullest information is in MacMunn's *The Spectroscope in Medicine*, but this was published in 1880 and requires extension, but very little in the way of correction, to bring it up to date. Cole's *Practical Physiological Chemistry* has been consulted for some wave lengths and includes a brief account of recent investigations in Cambridge on the chemistry and physiology of the blood pigments, which has been abstracted. In Wells' *Chemical Pathology* portions of the subject are treated more fully than by the writer, and the reader is referred to that book for further references (*e.g.*, under hæmatinæmia). Von Jaksch's *Clinical Diagnosis* (translated by Garrod) gives several points not easily found elsewhere.

#### NAKED-EYE APPEARANCE

THIS should always be noted carefully, for sometimes it may give valuable indications. Normally, after centrifuging, the corpuscles occupy half or a little more than half the total volume. Marked constriction of the arm when drawing the blood causes the proportion of corpuscles to increase. After severe hæmorrhage, or in any marked anæmia, the proportion of corpuscles is of course reduced. Prolonged constriction of the vein is the commonest cause of the blood appearing very dark (see "reduced hæmoglobin"). A rare cause is polycythæmia. Sometimes the whole blood as drawn has a creamy sheen, and the plasma or serum should always be examined to see whether lipæmia is present.

#### LIPÆMIA

The term "lipæmia" should strictly only be applied when the *fresh* plasma is milky. Plasma on standing overnight often becomes opalescent, that is not true lipæmia. Normal plasma, obtained after a night's fast, is clear although it contains a total of 450 to 1,260 mgm. "fats" or lipoids per 100 c.c. (see p. 334). With larger quantities of fats in suspension the plasma becomes increasingly opalescent until with a gross excess it is milky, hence lipæmia of different grades. Lipæmia quite commonly occurs in healthy individuals a short time after a meal rich in fat, and is then physiological. Pathological lipæmia occurs in some cases of diabetes mellitus, of xanthomatosis, and of chronic parenchymatous nephritis (nephrosis). Often in these cases the lipæmia persists after a night's fast.

## COLOUR

The colour of the plasma or serum may give useful information. Normally it is pale yellow or straw, and is due mainly though not entirely to bilirubin (see Icterus Index, Chapter XII). In jaundice the plasma or serum is obviously a deeper yellow owing to the hyperbilirubinæmia. In carotinæmia the abnormal yellow colour of the plasma is due to lipochromes, carotin or xanthophyll. The commonest cause of carotinæmia is excessive ingestion of vegetables (green vegetables, carrots, egg yolk, etc.). The condition has also been termed xanthochromia and xanthosis. It is not uncommonly noted in the initial stages of the treatment of diabetics with diets rich in vegetables. It may occasionally be encountered in patients who are mobilising large quantities of fat from their depots. The latter factor may coexist with the diet factor in some of the examples seen in diabetics. Carotinæmia may be encountered in non-diabetic patients who are not eating many vegetables. It may occur in any cachexia.

Hyperbilirubinæmia may readily be distinguished from carotinæmia by Van den Bergh's test (see Chapter XII). Bilirubin gives a pink colour with the diazo reagent, the lipochromes do not. Both are included in the Icterus Index (see Chapter XII). Spectroscopically lipochromes give no absorption of the colours, whereas solutions of bilirubin give a continuous absorption of the blue end of the spectrum, but no absorption bands.

In addition to carotinæmia, there are other rare conditions in which "false jaundice" is seen, e.g., after the ingestion of picric acid—a device occasionally used in the Great War by malingerers.

Reddish tinges are generally due to hæmolysis occurring after the blood is drawn. This may easily be checked by examination with a direct vision spectroscope for the characteristic bands of oxyhæmoglobin. Occasionally, however, true hæmoglobinæmia, i.e., hæmoglobin present in the plasma *in vivo*, does occur (see later).

The whole blood may be chocolate in colour, and the plasma brown or normally coloured according to the location of the pigment, in methæmoglobinæmia and sulphæmoglobinæmia (see p. 328). In carboxyhæmoglobinæmia the blood is pinker than normal, especially when diluted (see p. 319).

On very rare occasions other tints may be observed. The writer once found the plasma yellowish brown in a woman who had been dosing herself for some time with extracts of enormous quantities of senna pods—two hundred and fifty pods daily! On spectroscopic examination the plasma showed no abnormal absorption bands. In a case of kala azar (untreated) in a child, the fresh serum was distinctly green—probably due to the formation of biliverdin.

## THE SPECTROSCOPICAL EXAMINATION OF THE BLOOD

A description of the use of the direct vision spectroscope has been given in Chapter X.

Blood is obtained by venepuncture, and serum or plasma is separated carefully in the usual way. The serum is examined directly with the spectroscope. The corpuscular mass is diluted in the centrifuge tube by 5, 10 and if necessary more volumes of distilled water, and after each addition the mixture is examined spectroscopically. When the bands of a foreign pigment are near those of oxyhæmoglobin the dilution has to be considerable to avoid

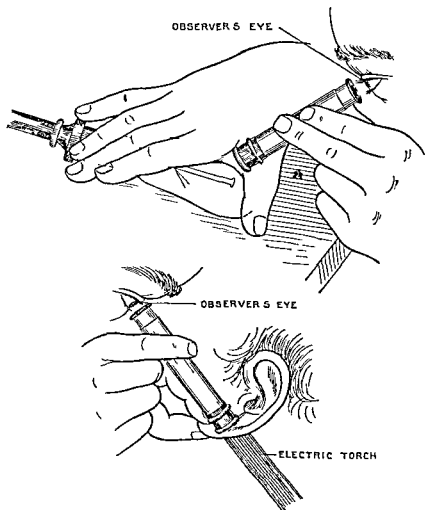


FIG 61 Spectroscopic examination of the blood *in vivo* in the web of the thumb and in the lobe of the ear

the masking effect of the latter, and it follows that in such cases the test is not sensitive. When, however, a foreign pigment yields absorption bands in different parts of the spectrum the spectroscopic test is fairly sensitive. Thus in methæmoglobinæmia and in sulphæmoglobinæmia the characteristic band in the red may be sought for in a dilution of 1 in 5 and 1 in 10 respectively when viewing a 1-in thickness of solution, or a thin layer of undiluted circulating blood may be examined in the web between the thumb

and first finger, or in the ear (see Fig. 61). The examination *in vivo* is not so delicate as the examination of the blood *in vitro*.

### HAEMOGLOBIN AND ITS DERIVATIVES

The absorption spectra are illustrated on p. 212, and the wave-lengths ( $\lambda$ ) of the different absorption bands are summarised below.

*Wave-lengths of maxima of absorption bands of hæmoglobin and its derivatives in tenth-metres, 0.1  $\mu$ , or Ångström units*

Substance	No of Bands	$\lambda$ of Absorption Bands.				Remarks
		$\alpha$	$\beta$	$\gamma$	$\delta$	
Oxyhæmoglobin . . .	2	5,780	5,400	—	—	5,768 and 5,398.†
Reduced hæmoglobin . . .	1	5,560	—	—	—	
Carboxy hæmoglobin . . .	2	5,720	5,350	—	—	5,714 and 5,360 †
Nitric-oxide hæmoglobin . . .	2	5,785	5,418	—	—	Hartridge †
Methæmoglobin . . .	4	6,300	5,780	5,400	5,000	
Alkaline methæmoglobin	2 (3)	(6,040)	5,779	5,406	—	<i>J. Physiol</i> , 1920, 54, 255
Sulphæmoglobin . . .	1 (3)	6,180	(5,780)	(5,400)	—	$\beta$ and $\gamma$ bands are in position of $\alpha$ and $\beta$ bands of oxy-Hæmoglobin
Acid hæmatin in ether . . .	4	6,380*	5,820	5,400	5,050	
Alkaline hæmatin . . .	1	6,100	—	—	—	In N/10 NaOH.
Hæmochromogen (globin)	2	5,585	5,275	—	—	
Acid protoporphyrin . . . In 25 per cent. w/v HCl	2 (3)	6,024	5,572	—	—	A fainter band between $\alpha$ and $\beta$ at 5,822 is also described
Alkaline protoporphyrin	4	6,452	5,910	5,397	5,040	In N/10 alkali.
Acid uroporphyrin . . .	3	5,966	5,776	5,536	—	} In 25 per cent. w/v HCl.
Acid coproporphyrin . . .	3	5,939	5,746	5,509	—	
Alkaline uroporphyrin . . .	4	6,120	5,605	5,390	5,037	In N/10 alkali
Alkaline coproporphyrin	4	6,175	5,685	5,385	5,039	In N/10 alkali.
Urobilin . . . . .	1	4,900	—	—	—	

\* The wave length of the  $\alpha$  band of acid hæmatin in N/10 HCl is about 6,620, and in acetic acid about 6,300.

† Hartridge, H., *J. Physiol*, 1920-21, 54, 258

Note. For wave lengths of Fraunhofer lines, see p. 210.

The work of the Cambridge school (Anson and Mirsky, Keilin, Hill and Holden) has made clear the relationships of hæmoglobin and its derivatives. An abstract of Cole's summary follows.

Reduced hæmoglobin is a compound of alkaline reduced hæmatin and native globin, the iron is in the ferrous state.

Oxyhæmoglobin is oxygenated (not oxidised) hæmoglobin, the iron is still in the ferrous state, the oxygen is held in loose combination.

Methæmoglobin is a compound of neutral hæmatin and native globin. It is oxidised (not oxygenated) hæmoglobin, the iron is in the ferric state.

Reduced hæmatin contains ferrous iron, and is  $C_{34}H_{32}N_4O_4Fe$ .

Hæmatin is formed by oxidation of reduced hæmatin, and contains ferric iron. It is  $C_{34}H_{32}N_4O_4FeOH$ .

Parahæmatin (kathæmoglobin) is a compound of neutral hæmatin and denatured globin.

Hæmin is hæmatin chloride,  $C_{34}H_{32}N_4O_4FeCl$ .

Hæmochromogen (globin hæmochromogen) is a compound of alkaline reduced hæmatin and denatured globin. It contains ferrous iron.

The porphyrins are pigments obtained from hæmatin by splitting off the ferric iron, or from reduced hæmatin by splitting off the ferrous iron.

Protoporphyrin (= Kammerer's porphyrin = blood porphyrin = coporphyrin) may be formed, by treatment with dilute hydrochloric acid, either from reduced hæmoglobin in the absence of oxygen, or from reduced hæmatin, it may also be prepared by treating blood with acetic acid and hydrazine. Protoporphyrin combines with iron to give hæmatin (protohæmatin). Four molecules of protohæmatin when reduced to "reduced hæmatin," and combined with the native protein globin give hæmoglobin (reduced hæmoglobin). All the natural hæmoglobins are derived from protohæmatin and therefore from protoporphyrin.

The following probably represents the chemical relationships of the porphyrins —

Reduced hæmatin,  $C_{20}H_4FeN_4$  ( $4 CH_3$ ,  $2 CH_2CH_2COOH$ ,  $2 CH=CH_2$ )  
 Protoporphyrin,  $C_{20}H_4N_4$  ( $4 CH_3$ ,  $2 CH_2CH_2COOH$ ,  $2 CH=CH_2$ )  
 Hæmatoporphyrin,  $C_{20}H_6N_4$  ( $4 CH_3$ ,  $2 CH_2CH_2COOH$ ,  $2 CHOHCH_3$ )  
 or  $C_{34}H_{38}N_4O_6$  is not found in nature. It is prepared in the laboratory by the action of concentrated  $H_2SO_4$  on blood or hæmin. It is unfortunate that it has been called "hæmatoporphyrin," because, in medicine, that term used to be employed for the porphyrins as a group in urine or in fæces respectively.

Mesoporphyrin,  $C_{20}H_6N_4$  ( $4 CH_3$ ,  $2 CH_2CH_2COOH$ ,  $2 CH_2CH_3$ ) or  $C_{34}H_{38}N_4O_4$ , is reduced protoporphyrin.

Uroporphyrin,  $C_{20}H_6N_4$  ( $4 CH_2COOH$ ,  $4 CH_2CH_2COOH$ ), is the chief porphyrin found in the urine of man pathologically.

Sterco- or copro porphyrin,

$C_{20}H_6N_4$  ( $4 CH_3$ ,  $4 CH_2CH_2COOH$ ),  
 is the porphyrin found in human fæces and normal urine.

They each contain four pyrrole rings, united by methene ( CH) bridges to form a porphyrin ring

Following the method of expressing these relationships in Peters and Van Slyke's *Quantitative Clinical Chemistry*, the group  $C_{34}H_{32}N_4O_4$  may be termed porphyrin and symbolised as Por and the hæmoglobin compounds and derivatives classified as follows —

#### *Iron free Compounds*

Porphyrin group, $C_{34}H_{32}N_4O_4$	Por
Protoporphyrin $C_{34}H_{34}N_4O_4$	Por $H_2$
Uroporphyrin, $C_{40}H_{38}N_4O_{16}$	Por $(COOH)_6$
Copro or sterco porphyrin, $C_{36}H_{38}N_4O_8$	Por $(COOH)_2H_4$

#### *Ferrous Compounds*

Reduced hæmatin	Por $Fe^{++}$
Reduced hæmoglobin	(Globin) (Por $Fe^{++}$ )
Oxy hæmoglobin	(Globin) (Por $Fe^{++}O_2$ )
Carboxyhæmoglobin	(Globin) (Por $Fe^{++}CO$ )
Nitric oxide hæmoglobin	(Globin) (Por $Fe^{++}NO$ )
Globin hæmochromogen	(Denatured globin) (Por $Fe^{++}$ )

#### *Ferric Compounds*

Hæmatin	Por $Fe^{+++}OH$
Hæmin, or hæmatin chloride	Por $Fe^{+++}Cl$
Methæmoglobin	(Globin) (Por $Fe^{+++}OH$ )
Cyanhæmoglobin	(Globin) (Por $Fe^{+++}CN$ )

### HÆMOGLOBINÆMIA

Normally hæmoglobin is confined within the corpuscles. The presence of hæmoglobin free in the plasma (hæmoglobinæmia) has been described in pernicious anæmia, in paroxysmal hæmoglobinuria, after the transfusion of incompatible blood and in blackwater fever. In the laboratory it is very commonly noted that the plasma or serum contains traces of hæmoglobin, but such hæmolysis has nearly always occurred after withdrawal of the blood. In fact, it is not easy to prevent this hæmolysis (see note under Icterus Index, Chapter XII)

### REDUCED HÆMOGLOBIN

Solutions of reduced hæmoglobin show a single absorption band between D and E (p 212). The dark colour of venous blood collected after applying constriction to the arm is due to reduced hæmoglobin. The proportion of reduced hæmoglobin in blood obtained from patients, however, cannot conveniently be determined by spectroscopic examination. It may be measured by the oxygen unsaturation of the blood. Lundsgaard has shown that about 5 gm of reduced hæmoglobin per 100 c c of capillary blood are necessary to cause cyanosis. This corresponds to an oxygen unsaturation of about 7 volumes per cent. The amount of oxyhæmoglobin present

has very little effect on the cyanotic hue (*cf* also Chapter XIX, under Oxygen)

### CARBOXYHÆMOGLOBINÆMIA AND CO-POISONING

Carboxyhæmoglobin is more stable than oxyhæmoglobin, so that if carbon monoxide is breathed for any length of time the carboxy-compound replaces oxyhæmoglobin. It is easy to understand, therefore, that air containing small proportions of CO is dangerous if breathed continuously for several hours. Frederick quotes the data of Henderson *et al*

Concentration	CO per cent
Allowable for an exposure of several hours	0 01
Can be inhaled for one hour without appreciable effect	0 04-0 05
Causing a just appreciable effect after one hours exposure	0 06-0 07
Causing unpleasant but not dangerous symptoms after one hour s exposure	0 1 -0 12
Dangerous for exposure of one hour	0 15-0 20
Fatal in exposure of less than one hour	0 4 and above

On the other hand, if the patient is removed to a CO free atmosphere and breathing is maintained, the CO is fairly quickly washed out. It is important to remember this when examining the blood from cases of suspected CO poisoning. If artificial respiration has been applied for some time previous to the drawing of the blood, and particularly if it has been combined with oxygen administration, the CO content of the blood examined may be very much less than the CO content of the blood at the time of the patient's collapse.

Up to 15 or 20 per cent of the hæmoglobin in a non anæmic person may be combined with CO before symptoms of CO poisoning are noted. It is not uncommon, for instance, for the blood of tobacco smokers to contain about 5 per cent of CO hæmoglobin. The quantity of oxyhæmoglobin left, however, is the important point, for symptoms only occur when the blood cannot carry sufficient oxygen. A statement of the percentage of carboxy hæmoglobin may, therefore, be misleading by itself. If the subject is already anæmic, for example, a smaller percentage than 20 would suffice to cause symptoms of CO poisoning. With an approximately normal total hæmoglobin, Henderson and Haggard relate the symptoms to the percentage saturation as follows —

Hæmoglobin combined with CO per cent of total Hb	Effect
10	No appreciable effect except shortness of breath on vigorous muscular exertion
20	No appreciable effect in most cases except short wind even on moderate exertion, slight headache in some cases
30	Decided headache, irritation, ready fatigue, disturbance of judgment
40-50	Headache confusion collapse and fainting on exertion
60-70	Unconsciousness respiratory failure and death if exposure is long continued
80	Rapidly fatal
Over 80	Immediately fatal.

The sources of carbon monoxide which may give rise to poisoning are numerous—coal gas, "fire damp" in mines, exhaust gases (*e.g.*,

from chimneys, motor cars and various combustion engines), the air of burning houses, of sewers, etc

### Recognition of, and Estimation of Degree of Carboxyhæmoglobinaemia

On boiling diluted blood containing CO hæmoglobin the coagulum is at first red, but on further boiling it turns brown

Carboxyhæmoglobin is not reduced as is oxyhæmoglobin, after death from CO the blood is bright red and the body has a pink tinge

Carboxyhæmoglobinaemia can be demonstrated colorimetrically owing to the fact that in high dilutions carboxyhæmoglobin is more pink than the corresponding dilutions of oxyhæmoglobin or spectroscopically with a reversion spectroscope, or by blood gas analysis In examining blood it is a question of recognising carboxy hæmoglobin in the presence of oxyhæmoglobin, and naturally the problem is more difficult when the proportion of carboxyhæmoglobin is small The pink tint of diluted blood (*eg*, about 1 in 300 in a test tube or boiling tube) is sufficient to establish the diagnosis in a case of coma, or after death due to CO poisoning, for the pink colour is marked on comparison with normal blood diluted to about the same degree But in lesser grades of CO poisoning it is necessary to work quantitatively (*cf* below) when comparing the patient's with normal blood, otherwise a slightly deeper colour due to a higher concentration of oxyhæmoglobin may be mistaken for the greater pinkness of a mixture of carboxyhæmoglobin and oxyhæmoglobin

Colorimetric methods include Haldane's using carmine solution, Sayers and Yant's employing tannic acid and pyrogallol, and the following simple clinical method

### Determination of the Approximate Percentage of Carboxyhæmoglobin in Blood

**Principle** The total hæmoglobin is determined by Haldane's CO method but in a Lovibond comparator (*see p 296*) instead of the usual hæmoglobinometer A second sample of the same volume of blood is diluted to the extent found in the previous determination but is not treated with CO, its colour is matched in the Lovibond comparator against the nine coloured glasses which correspond to 0 10 20 30, 40 50 60 70 and 100 per cent carboxyhæmoglobin and the reading is taken

**Method** Place dilute ammonia solution (0.4 c.c. of concentrated ammonia solution S.G. 0.88 in water to 100 c.c.) in a graduated Lovibond comparator tube up to the mark 20 c.c. (which correspond to 20 per cent on the Haldane scale) Measure accurately 0.04 c.c. of blood with the pipette specially provided (marked at 0.02 and 0.04 c.c. and calibrated to contain) wiping off all blood from the exterior, deliver it into the dilute ammonia solution and wash it out with the same Add a minute drop of caprylic alcohol saturate with coal gas and dilute with more ammonia solution in steps until it matches exactly the 100 per cent COHb glass After each addition of ammonia solution pass coal gas in order to saturate and to mix thoroughly As usual it is best to note the reading when the dilution is not quite sufficient and again when it is just too great and then to decide on the intermediate point at which the match is considered to be perfect Suppose the final value corresponds to 8.0 c.c. the total hæmoglobin is then 80 per cent, on the Haldane scale

Now measure another 0.04 c.c. of blood into about 2 c.c. of ammonia solution in a graduated comparator tube Dilute it with ammonia solution to exactly the same extent (8.0 c.c. in the example quoted) but do not pass any coal gas Compare the



colour of the diluted blood with the glasses in the disc. If there is no carboxyhaemoglobin it will match the 0 per cent COHb glass (= 100 per cent oxyhaemoglobin). There may be some doubt when the blood contains 10 per cent or less COHb, but none with 20 per cent or more. Read off directly the percentage of the total haemoglobin which is carboxyhaemoglobin. Since the total haemoglobin is known it is easy to calculate the grammes of COHb and  $\text{HbO}_2$  per 100 c.c. of blood. Thus

Total haemoglobin	= 80 per cent (Haldane scale)
	= $80 \times 0.138^1$ gm. per 100 c.c.
	= 11.04 gm. per 100 c.c.
Suppose COHb	= 30 per cent of total haemoglobin
Then COHb	= 3.312 gm. per 100 c.c. blood
$\text{HbO}_2$ (+ Hb)	= 7.728      "      "      "
Total Hb	= 11.040      "      "      "

**Remarks** The 0 per cent COHb glass (= 100 per cent  $\text{HbO}_2$ ) was prepared by matching blood ( $\text{O}_2$  combining power 18.5 c.c.  $\text{O}_2$ , Fe content 46.2 mgm. per 100 c.c.) diluted 1 in 250 and placed in a comparator tube. The 100 per cent COHb glass was similarly prepared after saturating the diluted blood with coal gas. The 10 per cent COHb (= 90 per cent  $\text{HbO}_2$ ) glass matched the colour of a pair of comparator tubes, one of which contained 1 c.c. of 1 in 250 blood and 9 c.c. of dilute ammonia solution and was saturated with coal gas, the other 9 c.c. of 1 in 250 blood and 1 c.c. of dilute ammonia but no coal gas, and so on.

The pipette is marked at 0.02 c.c. as well as at 0.04 c.c. in case the total haemoglobin should be very high or the volume of blood available very small. Naturally, when 0.02 c.c. of blood is used the total haemoglobin reading must be multiplied by 2.

The same principle can be employed without using a Lovibond comparator and disc, the COHb +  $\text{HbO}_2$  paired standards being freshly prepared from normal blood on the day of each test, tubes of standard bore and a comparator e.g. Cole and Onslow's are of course necessary. The technique originally was worked out in this way but clearly the use of coloured glasses as the standards saves a great deal of time and makes for uniformity.

Difficulty may be experienced in applying this technique to stale blood as, for instance, obtained more than twenty four hours post mortem, such blood is sticky and may contain clots and it is difficult to take true samples, also chemical changes in the oxyhaemoglobin may have occurred. Blood obtained up to about twenty four hours after death has served perfectly for the test.

Spectroscopically the absorption bands of carboxyhaemoglobin are very similar to those of oxyhaemoglobin (see p. 212), but are shifted slightly (about  $60 \text{ \AA}^\circ$ ) towards the violet end of the spectrum. It is therefore impossible to detect COHb in the presence of  $\text{HbO}_2$  with the simple direct vision spectroscope. Hartridge's reversion spectroscope, however, may be used not only for the detection, but also for an estimation. The following instructions are for the instrument supplied by Messrs. Bellingham and Stanley Ltd., but with slight modifications apply to any pattern.

### Detection and Estimation of COHb by Reversion Spectrometer

**Dilution of Blood for Test Purposes** The blood is diluted until the distance between the  $\alpha$  and  $\beta$  bands is about equal to the width of the  $\alpha$  band. This corresponds to a dilution of 1 part of normal blood in about 300 parts of diluent when the tube containing the diluted blood is about 1 in. in diameter. As diluent, dilute ammonia solution (0.4 c.c. of concentrated ammonia (S.G. 0.88) in water to 100 c.c.) is satisfactory because it prevents any precipitation of plasma proteins on dilution or on the subsequent passage of coal gas.

<sup>1</sup> 100 per cent (Haldane scale) = 13.8 gm. Hb per 100 c.c. = 18.5 c.c.  $\text{O}_2$  per 100 c.c. (oxygen combining power) = 46.2 mgm. Fe per 100 c.c.

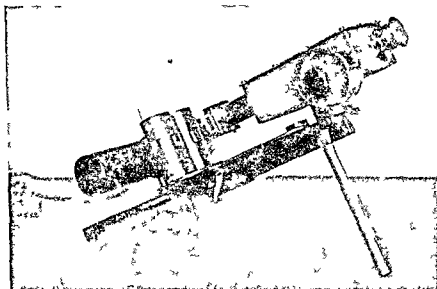


FIG 62 Reversion spectrometer mounted on viewing stand which carries the light source and a holder for two boiling tubes<sup>1</sup> the holder moves laterally so that first one and then the other tube can be brought in front of the spectroscope it may also be rotated through 90 degrees so that the light passes through both tubes it is easily removed so that glass cells can be substituted

**Qualitative Test for Carboxyhæmoglobin** Normal blood is diluted as above described and placed in the reversion spectrometer (Fig 62) The instrument is then set so that the  $\alpha$  bands of the two spectra of the oxyhæmoglobin exactly overlap (Fig 63A)

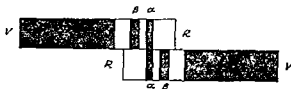


FIG 63A Instrument set so that the  $\alpha$  bands of the two spectra of  $\text{HbO}_2$  are colinear

The patient's blood is then similarly diluted and substituted for the diluted normal blood care being taken to avoid upsetting the adjustment of the instrument

If the patient's blood contains carboxyhæmoglobin a slight shift towards the violet occurs in the position of the bands so that the  $\alpha$  bar is no longer overlap exactly (Fig 63B)



FIG 63B Showing shift towards violet when diluted blood containing COHb is substituted for the  $\text{HbO}_2$  solution

<sup>1</sup> The viewing stand was designed by Mr F Stanley and the writer and the complete apparatus may be obtained from Messrs Bellingham and Stanley Ltd 71 Hornsey Rise London N 19

When used in the straightforward manner above described, the test is a rough one and will detect 50 per cent or higher saturation of hæmoglobin with CO

The method may be rendered more sensitive by carrying it out in a dark room, and with special filters. As before, the instrument is set with diluted normal blood, the  $\alpha$  bands of the two spectra being made colinear by means of the micrometer screw, and the mean of a number of readings on the drum being taken. The diluted blood of the patient is substituted and the mean reading again secured. In this way a 20 per cent and even a 10 per cent saturation with CO may be revealed. It is a question of making sure of slight shifts in the position of the absorption bands. When a sample is completely saturated with CO, the shift amounts to about six drum divisions (varying slightly with different instruments).

**Quantitative Estimation of Percentage Saturation with CO.** The readings should be made in a dark room.

Two special cells, A and B (Fig. 64), are provided which are of exactly the same thickness internally, *e.g.* about  $\frac{1}{2}$  in. or 12.5 mm<sup>1</sup> and are placed face to face. The instrument is used in a horizontal position, with the tube holder removed. The reducing wedge over the slit is adjusted and the eyepiece focussed, both are left without further adjustment until all readings are completed.

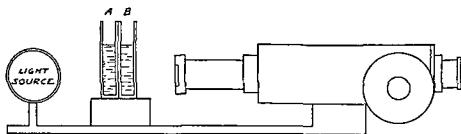


FIG. 64 Diagram to show relative positions of light source, the two cells and reversion spectrometer

Cell A is filled with distilled water. When everything else is ready, the patient's blood is diluted and put in cell B, and, as before, the  $\alpha$  bands are brought into coincidence and the mean of a series of readings taken.

It is essential to dilute sufficient blood to provide for this initial reading and for the preparation of the following standards (*e.g.*, dilute 1 c.c. of blood with the ammonia solution to 150 c.c.)

The 0 per cent CO standard is prepared by passing oxygen from a cylinder through the 1 in 150 blood in bright daylight for a few minutes, it is placed in cell B, and water in A.

The 100 per cent CO standard is prepared by passing coal gas in the dark through the 1 in 150 blood for a few minutes, it is placed in cell B, and water in A.

Intermediate standards are made as follows —

Standard	In Cell A		In Cell B	
	c.c. 1/150 Blood Saturated with Coal gas	c.c. of Ammonia Solution	c.c. 1/150 Blood Saturated with Oxygen	c.c. of Ammonia Solution
25 per cent CO .	5	15	15	5
50 per cent CO .	10	10	10	10
75 per cent CO .	15	5	5	15

In each case the final dilution is again treated with coal gas or oxygen respectively to make sure that it is saturated.

The mean reading for each of these standards is secured. When plotted the

<sup>1</sup> Provided the two cells are exactly similar their thickness is immaterial, for compensation can be effected by correspondingly greater or less dilution of the blood.

results for the five standards constitute a calibration curve from which the CO saturation of the patient's blood can be read off. It is not necessary to repeat the whole of this calibration for a given instrument every time a new blood is studied. If the 0 and 100 per cent standards are prepared each time and the instrument is adjusted so that these give the same readings as those utilised for the construction of the calibration curve, then intermediate readings can be converted into saturation per cent CO with the aid of the curve.

For the methods of estimation by blood-gas analysis the reader is referred to Peters and Van Slyke's *Quantitative Clinical Chemistry*, Vol II.

### NITRIC-OXIDE HÆMOGLOBINÆMIA

Solutions of nitric oxide hæmoglobin give an absorption spectrum very like that of oxyhæmoglobin, but the bands are less sharply defined and are situated slightly towards the red (see p 315). The bands, like those of CO-Hb, are unaffected by reducing agents. On dilution with water NO hæmoglobin gives a tint which is pinker than the corresponding dilution of oxyhæmoglobin, but which is not so pink as the corresponding dilution of CO hæmoglobin. On boiling diluted blood containing NO hæmoglobin the coagulum is pink, and it remains pink on further boiling (contrast CO hæmoglobin).

Banham, Haldane and Savage have reported a case in which NO hæmoglobin was formed after death from broncho pneumonia. They suggested that an organism producing nitrite must have been present. The nitrite reacted with the reduced hæmoglobin, and the resulting nitric oxide combined with more reduced hæmoglobin to form NO hæmoglobin. They point out that the possible presence of NO hæmoglobin in cases of suspected CO poisoning should be remembered.

The blood in NO hæmoglobinæmia has a striking pink colour. Banham noted that in many deaths from influenzal pneumonia the bodies had a red colour, and the blood was red or cherry red, though CO poisoning could be excluded. It is possible therefore that NO hæmoglobinæmia may be of clinical interest.

### METHÆMOGLOBINÆMIA AND SULPHÆMOGLOBINÆMIA

It is convenient to consider these together, for clinically the two conditions have much in common, and in the laboratory many tests are common to both, special methods being required for differentiation. Furthermore, it may be merely fortuitous whether the abnormal pigment be methæmoglobin or sulphæmoglobin, it having been suggested that a suitable catalyst (drug, drug product, toxin absorbed from the gut, or toxin liberated in the circulation) will cause methæmoglobin to be formed in the absence, and sulphæmoglobin to be formed in the presence of absorption from the gut of traces of sulphide. The fact that  $H_2S$ , in very small doses, kills by its action on the nervous system, does not rule out this hypothesis. In a few cases first one and later the other pigment has been demonstrated in the blood.

The following is a classification of the conditions in which methæmoglobinæmia and sulphæmoglobinæmia may occur

Methæmoglob æmia	Sulphæmoglobinæmia	Remarks
(a) Inhalation or absorption of coal tar derivatives (b) Drugs (excluding (g) ) (c) Intestinal stasis	(b) Drugs (c) Intestinal stasis	Pigment intracorpuseular No pigment in urine No hæmolysis
(d) Severe sepsis (e) Blackwater fever (f) Transfusion of incom patible blood (g) Phenylhydrazine poisoning	(d) Severe sepsis	Pigment extracorpuseular Methæmoglobin present in urine (Sulphæmoglobinuria in (d) has not been reported) Hæmolysis present

Industrial poisoning by coal tar derivatives, *eg*, in the manufacture of aniline dyes and certain explosives, is due to absorption through the lungs or the skin or by the gut, the substances responsible include aniline, nitrobenzenes and nitrophenols. Shoe dye poisoning (*cf* Levin) may be placed in the same category.

The drugs which have been reported as the cause of methæmoglobinæmia include acetanilide, antipyrine or phenazone, phenacetin, plasmochin, nitrites, potassium chlorate, prontosil, trional and sulphonal. Those responsible for sulphæmoglobinæmia include acetanilide ('Bromo Seltzer,' *cf* Harrop and Waterfield), methyl acetanilide ('Exalgin'), phenacetin (*cf* Snapper), prontosil (*cf* Discombe) and pyridium. Some people are peculiarly susceptible to the drugs or chemical compounds mentioned, and show the effects of poisoning after quite small doses.

Constipation, often marked, is present in many cases of methæmoglobinæmia and sulphæmoglobinæmia, and in the earlier reports was regarded as an important factor. It was thought that it favoured the absorption of some bacterial toxin or product which converted some of the hæmoglobin into the abnormal pigment, hence the terms 'enterogenous,' 'toxic' or 'microbic' cyanosis, 'idiopathic cyanosis' has also been employed to indicate that lesions of heart or lungs are absent. A nitrite forming bacillus was found in the saliva (*cf* Wallis) or in the fæces (*cf* Garrod) of some cases of sulphæmoglobinæmia, but has not been isolated by several other investigators (*cf* Vogel). Some of these patients were undoubtedly taking drugs of the coal tar series ('antikamnia tablets,' acetanilide or phenacetin), and of recent years the view that drug taking is the responsible factor in probably all the cases has received increasing support, the fact that there is no history of self medication is of doubtful value.

In the above examples the abnormal pigment is intracorpuseular, there is none in the plasma or urine. In the remaining groups the pigment is extracorpuseular, methæmoglobin passes into the urine (*cf* Chapter X), but so far as the writer is aware sulphæmoglobinuria

has not been definitely established, though it might be expected in extracorporeal sulphæmoglobinæmia, which has been reported

In severe sepsis, particularly septicæmia due to *B. Welchii*, both plasma and urine may be deep brown. Van den Bergh and Engelkes have described hæmolytic methæmoglobinæmia in a few cases of anaerobic sepsis and eclampsia, and hæmolytic sulphæmoglobinæmia in a few cases of anaerobic sepsis. Banham, Haldane and Savage suggest that methæmoglobinæmia may be responsible, in part at any rate, in certain cases of infective broncho pneumonia, for the cyanosis, which is peculiar in that it is not removed by the proper administration of oxygen. They further suggest that in these cases the methæmoglobin may be formed by the action of nitrites produced by nitrifying bacteria.

In blackwater fever, too, the plasma and urine may be intensely brown due to "methæmoglobin."

According to Fairley and Bromfield the brown pigment in the urine is methæmoglobin whereas that in the plasma is not true methæmoglobin but a new pigment "pseudo methæmoglobin" its  $\alpha$  band is removed by  $\text{Na}_2\text{S}_2\text{O}_4$  but not by Stokes' reagent ammonium sulphide or ammonia and is situated between those of sulphæmoglobin and true methæmoglobin (cf p 397). The same workers have produced pseudo methæmoglobin *in vitro* by incubating solutions of oxyhæmoglobin, methæmoglobin or sulphæmoglobin at  $40^\circ\text{C}$  with 4 volumes of sterile plasma. They suggest that oxyhæmoglobin in the plasma is partly converted into pseudo methæmoglobin (a protective mechanism) partly excreted unchanged and partly altered in the kidneys into true methæmoglobin. They therefore consider that the urinary methæmoglobin is formed in the kidneys and is not due to escape from the plasma.

Extracorporeal methæmoglobinæmia has been found after the transfusion of incompatible blood,<sup>1</sup> and after large doses of phenylhydrazine hydrochloride.

The clinical signs vary with the concentration of pigment and the condition responsible for the same. Cyanosis is the rule, in the intracorporeal group it is often of a peculiar violet hue, limited in the milder cases to the lips, ears and finger tips, but becoming general in the more marked examples and changing in severe types to a dusky leaden blue. In the extracorporeal group the cyanosis again varies and may even be masked by the brown coloration, thus in anaerobic septicæmia the patient may be copper coloured like a Red Indian. It is the cyanosis, unaccompanied by physical signs of disease in the heart or lungs, which usually leads to an examination of the blood for abnormal pigments. Presumably cyanosis becomes evident when about 5 gm of hæmoglobin per 100 c.c. of capillary blood are in the form of reduced hæmoglobin plus methæmoglobin or sulphæmoglobin (cf remarks above under "reduced hæmoglobin"). Dyspnoea is absent in the mild, present on exertion in the moderate, and present at rest in the severe cases, as would be expected from the corresponding diminution of the oxygen carrying power of the blood. Clinically also constipation, and varying degrees of headache and weakness are commonly noted.

<sup>1</sup> Dr Fairley writes that in the one case of incompatible transfusion so far tested (June, 1937) the plasma contained pseudo methæmoglobin.

### Tests Common to Methæmoglobinæmia and Sulphæmoglobinæmia

An absorption band in the red may be observed in the circulating blood *in vivo* (see p 314). Venous blood is obtained, oxalated and centrifuged. The separated plasma is examined directly with the spectroscope, before declaring the result negative as thick a layer as possible should be viewed in a tube from above (*cf* p 210). The corpuscles are laked with 5-10 and if necessary more volumes of water and examined spectroscopically at each stage. Owing to the presence of a relatively large amount of oxyhæmoglobin only the  $\alpha$  band in the red of the abnormal pigment is visible (*cf* chart of spectra facing p 212).

According to Bloem the spectroscopic test (1 in thickness of whole blood diluted 1 in 5 and 1 in 10 respectively) becomes positive with about 0.3 per cent methæmoglobin and about 0.52 per cent sulphæmoglobin, the corresponding limits for a 1 in thickness of plasma are about 0.06 and 0.05 per cent respectively.

The colour of the whole blood depends on the concentration of pigment, and is normal in mild, but tinted brown or chocolate in advanced cases, the plasma is brown in marked examples of the extracorporeal group.

Quantitative estimations (Stadie, McElroy, Van Slyke and Hiller) necessitate separate determinations of the total hæmoglobin colorimetrically or gasometrically, and of the oxyhæmoglobin gasometrically, the difference giving the concentration of abnormal pigment.

**Differentiation between Methæmoglobinæmia and Sulphæmoglobinæmia.** On the addition of a reducing agent (yellow ammonium sulphide, Stokes' reagent, or solid sodium hydrosulphite  $\text{Na}_2\text{S}_2\text{O}_4$ ) the band in the red disappears if methæmoglobin, but remains if sulphæmoglobin be responsible (Spectroscopically,  $\text{Methb} \rightarrow \text{HbO}_2 \rightleftharpoons \text{Hb}$ ). The  $\alpha$  band of methæmoglobin, but not of sulphæmoglobin, likewise disappears on adding concentrated ammonia solution (about 2 drops to 5 c.c.), owing to conversion to alkaline methæmoglobin which has two ill defined absorption bands in the green (Fig 47, p 212, *cf* also p 214). With 1 per cent potassium cyanide the spectrum of methæmoglobin changes in a few minutes to that of cyanhæmoglobin (which closely resembles that of reduced hæmoglobin), whereas the spectrum of sulphæmoglobin is only slowly altered (twenty four hours at room temperature).

The reversion spectroscope may be used not only for differentiation, but also for identification with an artificially prepared solution



FIG 65A. The reversion spectroscope has been set with the patient's diluted blood so that the  $\alpha$  bands overlap.

of methæmoglobin or sulphæmoglobin respectively, or for measuring the wave length of the  $\alpha$  band. Thus suppose the instrument is set with the patient's blood, suitably diluted, so that the  $\alpha$  bands of the two spectra are colinear (Fig 65A). Without altering the setting, a solution of methæmoglobin (1 in 100 normal blood plus a crystal of potassium ferricyanide) and a solution of sulphæmoglobin<sup>1</sup> (about 10 c.c. of 1 in 100 normal blood, 0.1 c.c. of 0.1 per cent aqueous phenylhydrazine hydrochloride and 1 drop of water saturated with  $H_2S$ ) are substituted in turn. With the one the  $\alpha$  bands are colinear,



FIG 65B A solution of methæmoglobin has been substituted. Clearly the patient's blood did not contain methæmoglobin.

with the other they obviously do not overlap, so that it is seen at once whether it is methæmoglobin or sulphæmoglobin that the patient's blood contains (Fig 65B). Moreover, should the patient's pigment be something else, neither of the two prepared solutions will show the  $\alpha$  bands overlapping exactly, this was the case with Fairley and Bromfield's new pigment (cf p 325), which they have termed "pseudo methæmoglobin". The wavelengths of the  $\alpha$  bands of these three hæmoglobin derivatives are, methæmoglobin 6,300, sulphæmoglobin 6,180, and "pseudo methæmoglobin" about 6,240 Å°.

The reversion spectroscopie enables another characteristic of sulphæmoglobin to be demonstrated clearly. On passing coal gas or CO the  $\alpha$  band of sulphæmoglobin is shifted towards the violet, whereas that of methæmoglobin is unaltered. This was discovered by Clarke and Hurlley with a simple direct vision spectroscopie, they showed that CO moved the band from 6,180 to about 6,130 Å°.

For spectra, see Fig 47, facing p 212.

It is interesting to record that in intracorporeal sulphæmoglobinæmias due to phenacetin and to prontosil, the writer and his colleagues have always found that the  $\alpha$  bands of the patients' pigments match exactly the  $\alpha$  band of artificially prepared sulphæmoglobin, this is noted because in some quarters the very existence of sulphæmoglobinæmia has been doubted.

### HÆMATINÆMIA

Schumm (1912) found hæmatin in the serum of a case of chronic acid poisoning. He also detected it in a few cases of malaria, anaerobic sepsis, eclampsia and pernicious anæmia. Van den Bergh demonstrated its presence in many, but not all, patients with pernicious anæmia, and in some cases of ruptured ectopic pregnancy. He never found it in secondary anæmia.

<sup>1</sup> Prepare the sulphæmoglobin solution just before it is used because it is unstable.



Hæmatinæmia is difficult to demonstrate with certainty, particularly when slight

### PORPHYRINÆMIA

Schumm (1916) found traces of a porphyrin in the blood in a case of congenital porphyria, and estimated the quantity as about 1 mgm per 100 c c. In clinical work much more information is obtained by examining the urine for porphyrin, which is difficult to detect in the blood

### BILIRUBINÆMIA

This has already been discussed (see Chapter XII) under Van den Bergh's test, Fouchet's test etc

### UROBILINÆMIA

Urobilin must be present in the blood plasma when urobilinuria occurs for there is no conclusive evidence that the kidney can form urobilin from bilirubin. The simplest way to detect it is by extraction of serum or plasma with acid alcohol. To 1 volume of serum (about 5 c c) 2 volumes of acid alcohol (1 per cent hydrochloric acid in ethyl alcohol) are added. The mixture is boiled for a few minutes, cooled and filtered. The filtrate is examined spectroscopically for the characteristic band at the junction of the green and blue (Fig 48 p 220). If bilirubin is present in the original serum the filtrate will be green or greenish blue owing to its oxidation to biliverdin but biliverdin gives no absorption band, so the spectrum of urobilin can readily be seen if urobilin is also there. If urobilin alone is present the filtrate will have a pinkish or pinkish yellow tinge but it is not safe to regard urobilin as responsible for these tints unless the absorption band is also evident. Blankenhorn has published a method for estimating urobilin in blood.

Urobilinæmia may be demonstrated in acholic jaundice, and in any hæmolytic jaundice when urobilinuria is marked, but urobilin is apparently a non threshold substance, so that tests of the urine are much more sensitive than tests of the blood. For this reason the blood is rarely examined for urobilin.

### SPECIAL QUALITATIVE TESTS

#### The Formol-Gel Test and the Aldehyde Reaction

Two drops of formalin (40 per cent formaldehyde) are added to 1 c c of serum and the mixture is allowed to stand for twenty-four to forty-eight hours at room temperature. In the vast majority of sera including all normal ones, nothing happens, but in a few the contents of the tube become completely jellified so that the tube may be inverted without spilling (Gaté and Papacostas). The test is not uncommonly positive in syphilis, and was at one time put forward as a simple substitute for the Wassermann reaction.<sup>1</sup> It has since been shown that the latter view cannot be maintained (cf Ecker)

Napier independently made a similar observation in kala-azar, in which disease, however, the reaction is much more marked. He recommended the addition of 1 drop of formaldehyde solution (his was 30 per cent) to 1 c.c. of serum. In untreated kala-azar the mixture becomes viscid at once, and sets in one or two minutes, so that the tube can be inverted, at the same time becoming whitish and opalescent. In three to twenty minutes the serum becomes absolutely solid and opaque. Napier found that gelation with formalin may also occur in phthisis, leprosy and malaria in half an hour or longer, but never observed the rapid formation of a solid opaque gel which is characteristic of kala-azar. For further details the reader is referred to Napier's interesting paper. A positive reaction has also been reported in trypanosomiasis (Rogers). Napier called his test The Aldehyde Reaction, partly because he found it was also given, though less readily, by acetaldehyde, and partly to stress the difference in intensity between his observations in kala-azar and those of Gaté and Papacostas in syphilis. He admits, however, that in all probability the variations in different diseases are simply variations in degree of a common reaction. The writer much prefers the older term, "formol gel test," as being more descriptive. In chemistry there are several "aldehyde reactions," and Napier's term suggests a test for an aldehyde, and gives no indication of the essential gelation of serum.

Oxalated plasma serves equally as well as serum for the test. Napier, from his observations in kala-azar, concluded that the reaction was in some way associated with the globulin fraction. The high plasma protein, with a greatly increased globulin, is striking in kala-azar (see p. 369), but significant variations in the plasma proteins of other diseases giving less marked formol gel reactions have not been established. The cause of the reaction is not yet known.

It is interesting to note that Napier has found the test so constantly positive in untreated kala-azar that he has largely substituted it for spleen puncture in his clinic.

Indicanæmia (see Chapter V)

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## CHAPTER XIX

### BLOOD ANALYSIS

- Books and General Articles** Peters and Van Slyke's *Quantitative Clinical Chemistry*, Vol I, Interpretations, Vol II, Methods  
Folin's *Laboratory Manual of Biological Chemistry*  
Myers' *Practical Chemical Analysis of Blood*  
De Wesselow's *Chemistry of the Blood in Clinical Medicine*  
*Composition and Physical Properties of Normal Human Blood* Gram,  
H C, *Am J Med Sc*, 1924 168, 511  
*Chemical Tests of the Blood Indications and Interpretation* Rockwood,  
R J *Amer Med Assoc*, 1928 91, 157  
*Micro-chemical Methods of Blood Analysis* King, E J, Haslewood,  
G A D, and Delory, G E *Lancet*, 1937, 1, 886

For convenience in reference the various constituents have been arranged alphabetically. A fairly complete list of the substances in normal blood, which have been estimated quantitatively, is given in the table which follows. A discussion of each constituent, with an account of the variations which are found in pathological conditions, would occupy too much space. The substances which are commonly estimated in clinical medicine will receive a fairly full description. For the rest, short notes, or no mention beyond that in the table, must suffice. In a few instances, a full discussion has already been given in a previous chapter, *e g*, bilirubin in Chapter XII. The references to substances already dealt with will be found in the last column of the table or in the Index. Results in this chapter are expressed in mgm per 100 c c, or c c of gas per 100 c c, or gm per 100 c c. For the calculations necessary to convert these values into millimols per litre, see Appendix.

The table of normal results is based largely on the literature, but partly on the writer's experience. It is important to know the *range* of variation in health and not merely the "average normal," for unless the result in a particular patient is definitely outside the normal range it cannot be regarded as pathological. The difficulty is that this "normal range" varies in some cases according to the method of analysis employed (see, for example, under Cholesterol, p 348), so that here and there the reader, using one method, may consider the tabulated range, which may have been based on several methods, too wide. Those workers who have been able to establish their own normal ranges by particular methods on large series of healthy individuals will not consult the table in those instances. But otherwise it should be a useful guide, and will at least lessen the risk of attaching pathological significance to a finding which is within normal limits—a mistake which has been made several times in clinical medicine.

In a few instances in the literature a statistical analysis has been made of the percentage distribution of results obtained in health with regard to certain ingredients of the blood. In this way the previous knowledge that the extremes of normal were so and so, but that the majority of analyses lay within a narrower range, has been put on a more exact mathematical footing, but the difficulty still remains that in interpreting the result in a particular individual it is always possible that he is one of the minority with findings close to the extreme limit of normal. As before, only figures clearly outside the normal range have definite significance, and probabilities, though they may influence the final conclusion, must be viewed with caution.

On the technical side, in several of the blood analyses the first step is the precipitation of the proteins by tungstic acid. It is convenient here to summarise in general terms this procedure, and to give variations in detail under each test.

#### Tungstic Acid Precipitation of Proteins.—

	<i>In whole- blood</i>	<i>In serum or plasma</i>
Distilled water	7 vols	8.0 vols
Blood or serum or plasma	1 "	1.0 "
10 per cent sodium tungstate ( $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ )	1 "	0.5 "
2/3 N sulphuric acid	1 "	0.5 "

In each case the filtrate is 1 in 10

Between 5 and 10 c.c. of blood must be collected for each of the great majority of blood analyses. Sugar or urea determinations, however, if made on capillary blood, need only 0.5 to 1 c.c.

*Blood Analysis Normal Results (mgm per 100 c c, unless otherwise stated)*

Estimation	Whole Blood	Plasma or Serum	Remarks
Acetone bodies, total (as acetone)	1 to 3	—	See Chapter IX.
Acetone and aceto acetic acid (as acetone)	0 to 1	—	
$\beta$ hydroxybutyric acid (as acetone)	0 to 3	—	
Albumin	—	3.4 to 6.7 per cent	3.8 to 5.2 by $\text{Na}_2\text{SO}_4$ and Kjeldahl (Salvesen)
Alkali reserve	—	53 to 77 vols $\text{CO}_2$ per cent	Infants, 46-63 (See Chapter IX and p 203)
Amino acid nitrogen	3 to 8	4 to 6.5	
Ammonia	0.1 to 0.25	0.1 to 0.25	
Amylase, diastase	—	3 to 10 Wohlgemuth units	See Chapter XIII
Bicarbonate (plasma) (mainly $\text{NaHCO}_3$ )	—	53 to 77 vols $\text{CO}_2$ per cent	See Chapter IX, and p 203
Bile acids	2.5 to 6.0	—	As glycocholic acid Method of Aldrich and Bledsoe
	—	5 to 12	As sodium glycocholate Szilard's method (see Chapter XII)
Bilirubin	—	0.1 to 0.5	Equals 0.2 to 1.0 Van den Bergh unit (see Chapter XII)
Bilirubin index, icterus index	—	1 to 6	Index of 1 corresponds to colour of 1 in 10,000 potassium dichromate (see Chapter XII)
Bromide (as bromine)	0.227 to 0.572	—	<i>Biochem J</i> , 1936, 30, 716
„ (as NaBr)	—	0.5 to 2.5	<i>Brit Med J</i> , 1936, ii, 957
Calcium (as Ca) total	5 to 7	0 to 11	} <i>J Biol Chem</i> , 1926, 71, 87
„ diffusible	—	3.6 to 4.6	
„ non diffusible	—	2.8 to 6.1	
Chlorides (as NaCl)	450 to 530	560 to 620	
„ (as chlorine)	270 to 320	340 to 380	
Cholesterol total	100 to 200	100 to 220	See also p 348
Corpuscular volume (haematocrit)	36 to 51 per cent	—	
Creatine	2 to 9	1 to 3	Majority (whole blood), 3 to 6.5
Creatinine	0.7 to 2	0.7 to 2	In "normal" hospital cases, up to 3.5 (see Chapter V)
Diastase, amylase	—	3 to 10 Wohlgemuth units	See Chapter XIII

*Blood Analysis : Normal Results (mgm. per 100 c c., unless otherwise stated)*  
—continued

Estimation	Whole Blood	Plasma or Serum	Remarks.
Ergothioneine (see Thioneine)	—	—	
Fats, "neutral" .	0 to 160	50 to 580	<i>J. Biol Chem</i> , 1935, 111, 613
"Fats," total . .	200 to 2,000	450 to 1,260	"Total lipoids" or "total ether soluble matter"
Fatty acids, total .	290 to 420	190 to 640	Fatty acids derived from glycerides, lecithin and cholesterol esters
Fibrin (ogen) .	110 to 210	200 to 400 (plasma)	
Fragility of corpuscles	0.48 to 0.42 per cent NaCl 0.38 to 0.32 per cent ..	. . .	Trace of hæmolyxis Average, 0.45 per cent. NaCl Hæmolyxis complete
Freezing point .	-0.51 to -0.62° C	-0.51 to -0.62° C	Average, -0.56° C
Globulins .	—	1.2 to 2.9 per cent	1.6 to 3.4 by Na <sub>2</sub> SO <sub>4</sub> and Kjeldahl (Salvesen)
Glucose (see "Sugar")			
Glutathione { Reduced Total	25 to 48 28 to 52	} Nil	<i>J. Biol Chem</i> , 1932, 97, 465, and <i>J. Clin Investig.</i> , 1934, 13, 963 Contains 10.4 per cent sulphur and 13.7 per cent nitrogen
Hæmoglobin . .	Men, 13 to 16 gm per 100 c c	—	Women, 12 to 14 gm. per 100 c c
" . .	Men, 95 to 120 " per cent "	—	Women, 88 to 102 per cent Haldane scale 100 " per cent " = 13.8 gm of hæmoglobin = 18.5 c c O <sub>2</sub> combining power = 46.2 mgm Fe per 100 c c
Hydrogen ion concentration	pH 7.3 to 7.5 5 to 3.2 mgm per 100,000 litres 0.000,005 to 0.000 0032 mgm per 100 c c 0.005 to 0.0032 γ per 100 c c	pH 7.3 to 7.5	(See Chapter IX) Mgm of ionic hydrogen (1,000 γ = 1 mgm)
Icterus index, bilirubin index	—	1 to 6	Index of 1 corresponds to colour of 1 in 10,000 potassium dichromate (see Chapter XII)
Indican . . .	—	0.03 to 0.08	<i>Deutsch Arch f Inn med</i> , 1916, 119, 177
Iodine { Total Alcohol insoluble	5 to 20 γ 1 to 6 γ	}	(1 γ = 0.001 mgm)

*Blood Analysis Normal Results (mgm per 100 c c , unless otherwise stated)*  
—continued

Estimation	Whole Blood	Plasma or Serum	Remarks.
Iron (as Fe)	40 to 55	0 06 to 0 22	Varies mainly with hæmo- globin content in whole- blood
Ketone bodies (see Acetone bodies)			
Lactic acid	5 to 35	—	May rise to 200 mgm during severe exercise
Lecithin (Lipoid P × 25)	250 to 450	170 to 350	
Lipoids (see Fats )			
Magnesium (as Mg)	2 to 4	1 to 4	Serum Mg generally 2 2 to 2 5
Nitrogen free in physical solution	1 2 c c per 100 c c of venous blood 1 3 c c per 100 c c of arterial blood 1 4 c c per 100 c c of blood saturated with air		
Nitrogen total	2 6 to 4 3 per cent	1 1 to 1 4 per cent	Average for whole blood, 3 per cent
„ protein	2 5 to 4 3 per cent	1 0 to 1 4 per cent	Protein N is about 99 per cent of total N of whole blood and more than 96 per cent of total N of plasma or serum
„ non protein	25 to 50	18 to 30	
Nitrogen distribution of non protein nitro- gen —			
Ambo acid N	3 to 8	4 to 6 5	
Ammonia N	0 1 to 0 2	0 1 to 0 2	
Creatine N	0 6 to 2 9	0 2 to 1	
Creatinine N	0 26 to 0 74	0 26 to 0 74	
Urea N	7 to 20	7 to 20	Up to 25 under 10 and over 60 years
Uric acid N	0 1 to 1 3	0 1 to 1 3	Usually 0 6 to 1 0
Rest or residual N	3 to 19	2 to 12	Average (whole blood) 11 mgm.
Rest N including amino acid N	6 to 28	5 to 18	
Oxygen (see Table on p 359)			
Phenols	2 to 8	—	J Biol Chem , 1918, 36, 99
Phosphatides (Lipoid P × 23 5)	—	60 to 335	J Biol Chem , 1935 111, 613
Phosphorus (as P) —			
Total	28 to 48	6 to 18	
Acid soluble	16 to 34	2 to 8	
Inorganic or free (adults)	2 to 5	2 to 5	Serum or plasma of adults usually 2 to 4, of children 4 to 6 mgm
Ester or organic Lipo l or lipin	14 to 29 8 to 18	0 to 4 3 to 14	



*Blood Analysis Normal Results (mgm per 100 c c, unless otherwise stated)*  
—continued

Estimation	Whole-Blood	Plasma or Serum	Remarks.
Potassium (as K)	150 to 250	18 to 21	
Proteins plasma (F + A + G)	—	5.8 to 8.6 per cent	6.3 to 8.0 by Kjeldahl (Salvesen)
Proteins, serum (A + G)	—	5.6 to 8.5 per cent	Average, about 7.0 per cent.
Fibrin (ogen) (F)	0.11 to 0.21	0.20 to 0.40 per cent	
Albumin (A)	—	3.4 to 6.7 per cent	Usually about 4 per cent.
Globulins (G)	—	1.2 to 2.9 per cent	Usually about 2 per cent.
Globulins as per centage total protein	—	20 to 45	
Ratio A : G	—	4 : 1 to 12 : 1	Usually about 2 : 1
Refractive index, at 17.5° C	—	1.349 to 1.351	Water = 1.333
Sodium (as Na)	170 to 225	325 to 350	
Solids total	18 to 25 per cent	8.5 to 10 per cent	
Specific gravity	1.041 to 1.067	1.024 to 1.038	S.G. of water = 1.000
Sugar fasting (capillary blood)	60 to 120	60 to 120	Increases slightly with age (see Chapter VII)
Sugar maximum after meals (capillary blood)	180	180	Increases slightly with age (see Chapter VII)
Sulphur (as S)—non protein —			
Total	3.84 to 5.06	3.11 to 3.86	} <i>J Biol Chem</i> , 1927, 73 623
Inorganic	0.28 to 0.65	0.50 to 1.12	
Ethereal	0.07 to 0.96	0.09 to 0.96	
Neutral	3.19 to 4.32	1.72 to 2.54	
Thioneine	0 to 5	0	Contains 14 per cent sulphur
Urea	15 to 40	15 to 40	Up to 50, under 10 and over 60 years
Uric acid	0.3 to 4.0	0.3 to 4.0	Usually 2 to 3
Urobilin	—	0.0 to 0.4	<i>J Biol Chem</i> , 1928, 80 477
Viscosity, at 20° C	3.6 to 5.4	1.7 to 2.0	Viscosity of water equals 1
Volume of blood	6 to 10 pints 3.5 to 7 litres  30 to 100 c c per 100 gm of body weight	3.5 to 6 pints 2 to 4 litres  4 to 6 c c per 100 gm of body weight	Average for whole blood 5.5, average for plasma 3 litres
Weight of blood — As percentage of body weight As fraction of body weight	7 to 9  1/14 to 1/11	4.3 to 5.7  1/23 to 1/18	Plasma averages about 5 per cent Plasma averages about 1/20

## CALCIUM

Serum or citrated plasma normally contains 9 to 11 mgm of total calcium per 100 c c. Of this calcium, part is diffusible and part is non diffusible. Furthermore, a portion is ionised and the rest is non ionised. There is at present however, no generally accepted method for the determination of ionic calcium and no general agreement as to the significance of diffusible calcium. In both instances the methods so far proposed are too complex for routine clinical work. In health about 2 mgm Ca are in simple physical solution about 4 mgm in combination with proteins and about 4 mgm per 100 c c are held in solution by unknown factors and possibly in virtue of the action of the parathyroids. There is little or no calcium in the red cells, and determinations in whole blood are of no clinical value. For a fuller discussion the reader is referred to Peters and Van Slyke's *Quantitative Clinical Chemistry*.

In different diseases the serum calcium may be normal, lowered or raised.

*Hypocalcæmia*

Tetany { Infantile (rachitic)  
After parathyroidectomy

Chronic azotæmic nephritis  
(interstitial) (advanced cases)  
Renal infantilism (some cases)  
Rickets (the minority of cases)  
Osteomalacia (some cases)  
Celiac disease (few cases)  
Sprue (few cases)  
Idiopathic stertorhœa  
Chronic sepsis (a few cases)

*Hypercalcæmia*

Administration of an excess  
of parathyroid extract  
Parathyroid tumours  
(generalised osteitis fibrosa)  
Multiple myelomatosis (some  
cases)

## The Clinical Value of Calcium Estimations

The chief value of calcium estimations is in the diagnosis of tetany, and particularly of infantile tetany. Occasionally the test is really essential but in most patients there is little doubt from the clinical examination whether the observed convulsions are due to tetany or not. The seizures in tetany, as a rule, do not begin till the serum calcium has fallen to 6 or 7 mgm per 100 c c, but values below 7 mgm may occur without such attacks. Convulsions due to other causes are associated with normal or only slightly lowered calcium figures. Not all types of tetany are associated with hypocalcæmia. In general the blood calcium is low in rachitic infantile tetany, tetany due to parathyroidectomy or tetany due to guanidine poisoning but normal in tetany due to hyperpnœa, to

gastric disease with vomiting, or to excessive dosage with bicarbonates

Calcium determinations are essential for the control of treatment by subcutaneous injection of parathyroid extract, gross hypercalcaemia (over 16 mgm), if maintained, is dangerous to life. A raised serum calcium is an important point in the diagnosis of generalised osteitis fibrosa, but is not absolutely diagnostic of that disease, a rise has been reported also, for instance, in some cases of multiple myelomatosis (distinguished by Bence Jones' proteinuria, p 28)

In rickets the serum calcium is generally within normal limits, but occasionally it is low, and is then not seldom accompanied by signs of tetany. Apart from those patients who exhibit tetany, or signs of increased neuro muscular excitability (Chvostek's sign, Trousseau's sign)—"spasmophilia," as it is often called—estimations of serum calcium are of little clinical assistance in rickets (see also p 365)

The influence of diseases of the kidney on the calcium of the serum has been discussed in Chapter V

In a fair proportion of cases of coeliac disease, sprue and idiopathic steatorrhoea of adults the calcium is lowered slightly to about 8 mgm, in a few cases it is definitely low, 5 to 8 mgm, but even then tetany is uncommon. The probable cause of the low values is defective intestinal absorption of calcium owing to the fatty diarrhoea and there is often no rise in the plasma inorganic phosphate (cf Fairley, Parsons)

A fall in the calcium percentage of the serum has been reported in a number of cases of chronic sepsis of various types, and administration of calcium salts has been claimed to be of value. Possibly the septic factor is responsible for the lowered values which are occasionally found in varicose ulceration. Otherwise, in skin diseases the serum calcium is almost always within normal limits. When making requests for analyses it should be remembered that normal values (total calcium) are the rule in urticaria, angio neurotic oedema, asthma, the purpuras, hæmophilia, pregnancy, eclampsia, jaundice of all types, and bone diseases (excluding rickets and generalised osteitis fibrosa)

Peters and Van Slyke (*Quantitative Clinical Chemistry*) emphasise the importance of a knowledge of the serum proteins and plasma inorganic phosphate when interpreting low serum calcium values. A coincident fall of serum proteins, as in nephrosis and in severe malnutrition is of itself sufficient to account for the low calcium by reduction of the fraction in combination with protein, the low protein and not the low calcium is the significant finding clinically. An increased inorganic phosphate, as in severe renal lesions must reduce the calcium. In short, as in the case of sugar, the calcium of the blood depends on a number of factors, gross departures from the normal generally may safely be interpreted in the light of the clinical findings alone, lesser variations may entail in addition a study of other ingredients of the blood (especially proteins and

inorganic phosphate), or metabolism experiment, or a study of the acid base balance of the blood, before they can be interpreted satisfactorily

When the calcium content of the plasma is normal, it appears to be impossible to raise it significantly by the oral administration of calcium salts, though Roe and Kahn maintain the contrary, provided that sufficient of the salt (5 gm of calcium lactate is recommended) is given without food (half an hour or longer before breakfast, or at least three hours after the last meal of the day) When the serum calcium is lowered, oral administration is well recognised as frequently of value as a therapeutic measure. Intravenous injections have to be made with great caution, and the rise in blood calcium produced thereby is of short duration. Obviously, when the cause of the calcium deficiency is known it is all important to treat the cause (*e g*, vitamin D (cod liver oil etc), and sunlight for rickets)

### Technical

In clinical work the method of Kramer and Tisdall is generally employed, and this is the method described below. Modifications have been introduced by Clark and Collip and by Trevan and Bambridge. Nephelometric determinations of calcium are generally regarded as satisfactory when applied to blood. For a general discussion of methods, see Peters and Van Slyke's *Quantitative Clinical Chemistry*, Vol II

**Principle** The calcium is precipitated from a known volume of diluted serum or citrated plasma by an excess of ammonium oxalate. The precipitate of calcium oxalate is separated by centrifuging, washed, dissolved in sulphuric acid and titrated with standard potassium permanganate. From the quantity of permanganate used in oxidising the oxalate, the amount of calcium is calculated.

**Technique** Into a tapered centrifuge tube which will hold 10 to 15 c c of fluid, place

- (1 or) 2 c c of serum,<sup>1</sup>
- 2 c c of distilled water,
- 1 c c of saturated ammonium oxalate

Mix well and allow to stand for thirty minutes<sup>1</sup> or longer

Centrifuge the tube at about 1,500 revolutions per minute for five minutes, or until the precipitate has packed down well. Remove the supernatant fluid by inverting the tube cautiously, noting whether any of the precipitate is disturbed. If it is, the tube must be righted again before any precipitate is lost, and recentrifuged. If the precipitate is undisturbed the inverted tube is allowed to drain for a minute or two and the mouth of the tube is wiped with a

<sup>1</sup> Thirty minutes is sufficient for complete precipitation of calcium as oxalate when using serum but at least eighteen hours is required for citrated plasma (*cf* Clark Herbert)

clean cloth or piece of filter paper. Alternatively, the supernatant fluid may be removed as completely as possible with a capillary pipette and test.

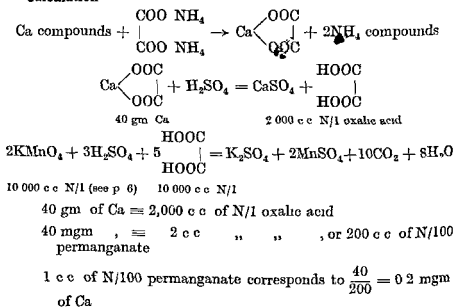
Add 4 c c of dilute ammonia (2 c c ammonia solution, S G 0.88 diluted to 100 c c), washing down the sides of the tube during the addition. Mix well and centrifuge for five minutes. Decant the supernatant fluid as before, and repeat the washing with 4 c c of the dilute ammonia, making two washings in all.

Add 2 c c of approximately N/1 sulphuric acid (28 c c of concentrated  $H_2SO_4$  water to 1,000 c c) to the precipitate, and place the tube in a boiling water bath for about one minute.

Titrate at once with 0.01 N potassium permanganate to a definite pale pink colour, which persists for at least one minute, using a 2 c c microburette graduated in fifths. Note the volume of permanganate required.

**Blank.** Place about 0.1 c c of the dilute ammonia in a centrifuge tube. Add 2 c c of the approximately N/1 sulphuric acid. Heat in the boiling water bath for about one minute, and titrate with the 0.01 N potassium permanganate. The blank value is usually about 0.02 c c of permanganate.

#### Calculation



Therefore (c c of 0.01 N permanganate minus the blank value)  $\times 0.2$  gives the number of mgm of calcium in the sample. Therefore when 2 c c of serum are used, the (titration less blank) multiplied by 10 gives the mgm of Ca per 100 c c of serum.

**Example of Calculation.** 2 c c of serum used. Titration = 1.10 c c of N/100 permanganate. Blank value = 0.025 c c of N/100 permanganate.

Therefore the permanganate used for the oxidation of calcium oxalate from 2 c c of serum =  $1.10 - 0.025 = 1.075 \text{ c c}$

1 c.c. of N/100 permanganate = 0.2 mgm. of Ca.

1.075 c.c. „ „ =  $0.2 \times 1.075$  mgm. of Ca

2 c.c. of serum contain  $0.2 \times 1.075$  mgm. of Ca.

$$\therefore 100 \text{ „ „ „ } 0.2 \times 1.075 \times \frac{100}{2} \text{ mgm. of Ca.}$$

$$= 1.075 \times 10 = 10.8 \text{ mgm. of Ca}$$

**Notes.** The 0.01 N. potassium permanganate is prepared afresh on the day of the test, by dilution of 0.1 N. permanganate, for the preparation of which see Appendix.

All glassware must be scrupulously clean. It is advisable to keep separately a number of tapered centrifuge tubes for this test only. After use they are washed out with water and then immersed in acid dichromate cleaning fluid (see Appendix) overnight. They are then thoroughly rinsed with water and cleaned in the usual way (see Appendix).

If a capillary pipette and teat is employed, care must be taken that the teat is quite free from calcium carbonate (rubber goods are sometimes stored in chalk, cf. p. 202). The teats used for this test should therefore be well rinsed with dilute hydrochloric acid, followed by water, then dried and stored separately.

The microburette must have a glass stop-cock. The "rubber and clip" type is useless for permanganate.

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results to state what has been done and whether whole-blood, plasma or serum has been used.

Ferguson and Roughton have brought forward evidence that not all of the  $\text{CO}_2$  in chemical combination is in the form of  $\text{BHCO}_3$ , but that some of it is combined with hæmoglobin as hæmoglobin-carbamino compounds.

*Normal  $\text{CO}_2$  Values \* (c.c. per 100 c.c., unless otherwise stated)*

	WHOLE-BLOOD.		PLASMA.	
	Min.	Max.	Min.	Max.
$\text{CO}_2$ capacity ( $\text{CO}_2$ in combination plus $\text{CO}_2$ in solution)	43 0	56 0	55 0	82 0
$\text{CO}_2$ combining power ( $\text{CO}_2$ in combination) or alkali reserve	41 0	53 0	53 0	77 0
$\text{CO}_2$ in physical solution (blood equilibrated with $\text{CO}_2$ at 40 mm.)	about 2.7		2 5	4 5
Arterial $\text{CO}_2$ content	32 0	60 0	—	—
"    "    " $\text{CO}_2$ in physical solution	1 5	4 0	—	—
"    "    " $\text{CO}_2$ in chemical combination	31 0	56 0	—	—
Venous $\text{CO}_2$ content	38 0	65 0	51 0	71 0
"    "    " $\text{CO}_2$ in physical solution	1 7	4 2	2 9	4 3
"    "    " $\text{CO}_2$ in chemical combination	36 0	61 0	48 0	67 0
Arterial $\text{CO}_2$ tension, mm.	22 0	63 0	22 0	63 0
Venous $\text{CO}_2$ tension, mm.	25 0	65 0	25 0	65 0

\* Compiled mainly from publications of Gram, Wright, and Peters, Barr and Rule.

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## CHLORIDES

The normal ranges in whole blood and in plasma are given on p 333, and the importance of securing "true plasma" for estimation of plasma chlorides has been emphasised in Chapter IX

*Hypochloræmia*

Intestinal obstruction  
 Mercury poisoning  
 Severe protracted vomiting  
 Severe burns  
 Pneumonia  
 Untreated diabetes (some cases)  
 Nephritis " "  
 Cholera " "  
 Fevers " "  
 Gross restriction of chloride intake  
 Addison's disease (*cf* p 378)

*Hyperchloræmia*

Nephritis (some cases)  
 Eclampsia " "  
 Prostatic obstruction (some cases)  
 Cardiac disease (some cases)  
 Severe anæmia " "

## Clinical Value

The estimation of blood chlorides, though of the greatest interest in special investigations such as of the distribution of the acid and basic radicles of the blood, until recently was not considered of much clinical assistance. Lately hypochloræmia has been discovered in many conditions in which there is loss of electrolytes owing to continued vomiting, gross watery diarrhoea, or marked exudations. This discovery has provided a rational explanation of the value of the saline injections which have been used empirically for many years. The chief value of chloride analysis is probably in intestinal obstruction and in patients with severe prolonged vomiting or severe burns, as a guide to treatment with sodium chloride (see Chapter IX). In nephritis (with or without œdema) the blood chlorides may be normal, low or high. In nephritis with œdema there is commonly a tendency to high values, but gross hyperchloræmia is unusual, presumably because water retention generally runs parallel with chloride retention (see Chapter V). Kidney disease with œdema may sometimes be associated with hypochloræmia. In "nephrosis" the blood chlorides commonly lie within normal limits. Though of little or no value in diagnosis the estimations are, however, some times of value as a guide to the degree of salt restriction (if any) which should be imposed in nephritis. It would appear to be unwise to restrict the salt intake when there is already hypochloræmia, or to continue unaltered the restriction when hypochloræmia has been produced.

The fact that in pneumonia the blood chlorides are low explains the diminished output in the urine in that disease, and leads to

the suggestion that the chlorides are "locked up" in the pneumonic exudate (see also Chapter XV)

The high figure for whole blood chlorides in severe *anæmia* may be due to the large proportion of plasma in the sample analysed

It has been suggested that the maintenance of the osmotic equilibrium of the blood depends largely on the chloride concentration, and that the fall in blood chloride which accompanies a rise in other crystalloids is a compensatory mechanism. On this basis may be explained the hypochloræmia of diabetes as due to hyperglycæmia, of advanced nephritis as due to retention of non protein nitrogen, and of alkalotic conditions as due to the increased plasma bicarbonate

There has been a general failure to confirm the suggestion of Allen that pure hypertension is due to an increase of the plasma chlorides

### Technical

Numerous methods for the estimation of chlorides in blood have been published, for an excellent discussion of which the reader is referred to Peters and Van Slyke's *Quantitative Clinical Chemistry*, Vol II. Whitehorn's method (*J Biol Chem*, 1920-21 45, 449) described below, is simple and convenient in that it can be applied to the tungstic filtrate obtained by Folin and Wu's method

**Principle** The proteins are precipitated by sodium tungstate and sulphuric acid. The chlorides in the protein free filtrate are precipitated by an excess of silver nitrate the excess being determined by back titration with potassium thiocyanate, using iron alum as indicator

### Solutions

(1) *Standard Silver Nitrate* Dissolve 4.791 gm of A.R. silver nitrate in about 100 c.c. of distilled water in a beaker, transfer quantitatively to a 1,000 c.c. volumetric flask and fill up to the mark with distilled water. The solution is 0.0282 N, and 1 c.c. corresponds to 1 mgm of Cl or 1.65 mgm of NaCl. It should be stored in a brown bottle

Alternatively, 5 c.c. of 0.033 N (N/30) silver nitrate (prepared by dilution of 0.1 N  $\text{AgNO}_3$ —see Appendix), or 10 c.c. of 0.02 N (N/50) silver nitrate may be used, in which case the following factors apply —

1 c.c. of N/30 silver nitrate = 1.18 mgm of Cl = 1.95 mgm of NaCl

1 c.c. of N/50 silver nitrate = 0.71 mgm of Cl = 1.17 mgm of NaCl

The thiocyanate employed must then similarly be prepared by dilution of a 0.1 N solution, and checked against the silver solution as described below

(2) *Potassium (or Ammonium) Thiocyanate (Sulphocyanide)* Because thiocyanates are hygroscopic, the standard solution must be prepared volumetrically. Dissolve 3 gm of KCNS or 2.5 gm of  $\text{NH}_4\text{CNS}$  in 1,000 c.c. of distilled water. Titrate 10 c.c. of the standard silver solution, to which about 5 c.c. of concentrated  $\text{HNO}_3$  have been added, with the thiocyanate delivered from a

burette, using iron alum as indicator. Note the volume required, and either dilute the thiocyanate so that 10 c.c. of it correspond exactly to 10 c.c. of silver solution, or label the bottle with the appropriate factor,  $x$  c.c. of thiocyanate = 1 c.c. of silver nitrate.

**Technique** Precipitate proteins as follows —

	<i>In whole blood</i>	<i>In "true" plasma</i>
Distilled water	14 c.c.	16 c.c.
Blood or plasma	2 "	2 "
10 per cent sodium tungstate ( $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ )—chloride free	2 "	1 "
2/3 N sulphuric acid	2 "	1 "

In each case the filtrate is 1 in 10

In a porcelain dish place

10 c.c. of protein free filtrate,

5 c.c. of standard silver nitrate solution,<sup>1</sup>

and stir thoroughly with a glass rod

Add 5 c.c. of concentrated chloride free nitric acid. Mix well and allow to stand for five minutes to permit the silver chloride to flocculate. With a spatula add about 0.3 gm. of solid finely powdered iron alum (ferric ammonium sulphate) and titrate the excess of silver nitrate with the standard thiocyanate solution from a 5 c.c. microburette, until the definite salmon red (not yellow) colour of the ferric thiocyanate persists in spite of stirring for at least fifteen seconds. Note the volume used.

**Calculation** Ten c.c. of protein free filtrate correspond to 1 c.c. of blood or plasma.

Five c.c. of the silver nitrate are used. Let the back titration with thiocyanate (of which 1 c.c. = 1 c.c. of silver solution) be  $x$  c.c. Then  $(5 - x)$  c.c. of the silver solution are required to precipitate the chloride in the 1 c.c. of blood. Therefore, since 1 c.c. of  $\text{AgNO}_3$  corresponds to 1 mgm. of Cl or 1.65 mgm. of NaCl the amount of chlorides in the blood equals

$$\begin{array}{l} \text{or} \quad 100 \times (5 - x) \text{ mgm. of Cl per } 100 \text{ c.c.} \\ \quad \quad 165 \times (5 - x) \text{ mgm. of NaCl per } 100 \text{ c.c.} \end{array}$$

**Example of Calculation from First Principles** 10 c.c. of filtrate from whole blood were used, and 5 c.c. of N/30  $\text{AgNO}_3$  were added. Back titration was 2.4 c.c. of KCNS, of which 9.6 c.c. corresponded to 10 c.c. of N/30  $\text{AgNO}_3$ .

$$9.6 \text{ c.c. of KCNS} = 10 \text{ c.c. of N/30 AgNO}_3$$

$$10 \text{ " " " " } = \frac{10}{9.6} \text{ " " " "}$$

$$2.4 \text{ " " " " } = \frac{10 \times 2.4}{9.6} = 2.5 \text{ c.c. of N/30 AgNO}_3$$

<sup>1</sup> Use 10 c.c. instead of 5 c.c. if the silver nitrate employed is N/50

Therefore  $\text{AgNO}_3$  used =  $5 - 2.5 = 2.5$  c.c. of N/30.

$\text{AgNO}_3$	+	$\text{NaCl}$	=	$\text{AgCl}$	+	$\text{NaNO}_3$
30,000 c.c. of N/30.		58.5 gm.				
30 c.c. of N/30.		58.5 mgm.				
1 c.c. of N/30.		1.95 mgm.				

Therefore 2.5 c.c. of N/30  $\text{AgNO}_3$  correspond to  $2.5 \times 1.95$  mgm. of  $\text{NaCl}$ .

Ten c.c. of filtrate represent 1 c.c. of whole-blood.

Therefore 1 c.c. of whole-blood contains  $2.5 \times 1.95$  mgm. of  $\text{NaCl}$ .

$\therefore$  Whole-blood chlorides equal  $2.5 \times 1.95 \times 100 = 488$  mgm. (as  $\text{NaCl}$ ) per 100 c.c.

### CHOLESTEROL

Cholesterol in the blood may be free or combined as esters. In the corpuscles the greater portion is free, in the plasma the greater portion is combined as esters. In clinical work, however, analysis is usually limited to a determination of the total (free plus ester) cholesterol, owing to the complexity of the differential estimation. The normal findings vary slightly with the method employed.

METHOD	Whole Blood mgm per 100 c c			Plasma or Serum mgm per 100 c c		
	Total	Free	Ester	Total	Free	Ester
Bloor <i>et al</i>	167 to 255	117 to 144	59 to 87	140 to 310	48 to 175	83 to 164
Myers & Wardell	100 to 200	—	—	100 to 200	—	—
Digitonin (Gardner & Gainsborough)	—	—	—	8 to 2.7	5 to 94	54 to 145
Van Slyke <i>et al</i>	—	—	—	118 to 360	56 to 121	61 to 201

The cholesterol content of the blood may be raised slightly a few hours (1 to 4) after food rich in cholesterol (particularly eggs, butter and meat), and may be lowered by a cholesterol poor diet (eggs meat, certain fish (salmon, bass), cheese, olive oil, etc, forbidden). In some cases it rises a little during pregnancy (rarely exceeding 400 mgm) to fall shortly after evacuation of the uterus. It may be raised a little during starvation.

#### *Hypocholesterolaemia*

Severe anaemia  
 Pernicious anaemia (some cases)  
 Severe sepsis  
 Severe cachexias

#### *Hypercholesterolaemia.*

Xanthomatosis  
 "Nephrosis"  
 Nephritis with oedema  
 Diabetes (some cases)  
 Arteriosclerosis (some cases)  
 Obstruction to bile passages, if complete or nearly so (Cholelithiasis, New Growth, etc)  
 Gout (some cases)  
 Sepsis (some cases)  
 Pregnancy (some cases)

Some of the most extreme examples of hypercholesterolaemia are met with in nephrosis (see Chapter V), the figures usually lie between 300 and 800 mgm per 100 c c. A portion of this cholesterol is in combination with the eu globulin fraction of the plasma proteins. Since the introduction of insulin, hypercholesterolaemia

has become less common in diabetes. Values up to 1,000 mgm have been reported, but most figures fall between 200 and 600 mgm. Some hold that diabetic hypercholesterolaemia is due to acidosis, others that it is due to the abnormal mobilisation of fat into the blood. Certainly hypercholesterolaemia may occur apart from acidosis. In diabetic xanthomatosis the excess of cholesterol is removed by adequate treatment with insulin, and the xanthoma deposits may disappear completely (Major). In the non diabetic cases hypercholesterolaemia (200 to 700 mgm) is the rule, but is absent in a few instances, in the non diabetic group insulin apparently has no influence on the cholesterol content of the blood or on the deposits (Ingram). Hypercholesterolaemia is usually a part of a general hyperlipidaemia.

### Clinical Value\*

Cholesterol estimations are useful in the diagnosis of xanthomatosis, and are often of great interest in the other conditions listed. The finding of hypocholesterolaemia is of little or no value clinically. It has been claimed that the prognosis in cases of enlarged prostate is better (that the operation risk is less) if there is hypercholesterolaemia, but this has not been confirmed by subsequent work. Likewise in diabetes cholesterol determinations have been regarded as of prognostic value but there is no general agreement on this matter. In nephritis with oedema the hypercholesterolaemia can almost always be prophesied from the clinical observations, blood tests are generally superfluous. In many other types of oedema (*e.g.*, cardiac) there is no excess of cholesterol, but there is generally no difficulty in distinguishing nephritic oedema from the other types on clinical grounds alone. In cancer there are no characteristic findings, slightly raised, normal and low figures have all been reported.

Since hypercholesterolaemia only occurs when obstruction of the bile passages is complete, or almost so cholesterol estimations are not of much value in the diagnosis of gall stones, etc (*cf* Chapter XII). On the whole, therefore, with the possible exception of xanthomatosis, determinations of the cholesterol content of the blood are of little help in clinical work, though they may be of the greatest interest in special investigations.

In pathological conditions the excess of cholesterol is generally more marked in the plasma than in the corpuscles. Examination of the whole blood, however, generally gives as definite information as examination of the plasma or serum, and is preferred for reasons of economy.

### Technical

Cholesterol may be determined colorimetrically or gravimetrically. The first method depends on the reaction of free plus ester cholesterol with acetic anhydride and sulphuric acid, the second on the precipitation of free cholesterol by digitonin (a

aponin) The first method may be carried out on 1 c c of blood, the second is less suitable for clinical purposes owing to the relatively large volume of blood, the time and the expense involved Schoenheimer and Sperry have evolved a micro method for free and total cholesterol in which the two principles are combined The *colorimetric technique of Myers and Wardell (total cholesterol)* is recommended and will now be described

**Principle** The blood is mixed with plaster of Paris and dried This makes the blood finely divided and readily extractable, and the calcium holds up certain substances which would otherwise give an additional colour subsequently The dried blood is extracted repeatedly with chloroform, and the extract is treated with acetic anhydride and sulphuric acid The resulting green colour is compared with that of a standard cholesterol solution similarly treated

**Method** In an evaporating basin of 3 in diameter place 4 to 5 gm of plaster of Paris (hemihydrate of calcium sulphate), and add 1 c c of whole blood, plasma or serum Stir thoroughly with a glass rod 3.5 in long Place the basin, with the rod inside, in a drying oven at 60° to 80° C for an hour or more At intervals break up the powder with the rod and mix the contents of the basin When a dry free flowing powder has been secured, scrape it out completely with a spatula or pocket knife on to a fat free filter paper (Whatman No 43) Transfer it quantitatively to a Soxhlet thimble (26 × 60 mm is convenient) Screw up the filter paper into a ball, which place on top of the powder in the mouth of the thimble, this breaks the fall of the drops of chloroform in the subsequent distillation and lessens the risk of the powder being splashed out of the thimble See that the top of the thimble is a few millimetres higher than the top of the syphon, thus making sure that no powder can be floated out of the mouth of the thimble—if necessary prop up the thimble to the required height by putting a bit of glass rod or copper wire or a pellet of fat free paper underneath Extract in the Soxhlet apparatus (Fig 66) for an hour or longer Then remove the thimble from the apparatus, which connect up again, and distil the chloroform into the thimble chamber Stop the distillation when the level of the chloroform is just short of the top of the syphon Disconnect the apparatus cautiously to avoid syphonage of the chloroform from the thimble chamber into the flask By this means



FIG 66 Soxhlet apparatus The flask contains chloroform which is vaporised and passes via the side arm into the condenser from which it drops into the thimble (not shown) in the middle chamber When the chloroform reaches the top of the syphon tube it runs back into the flask to be vaporised again but leaves behind the cholesterol etc, it had dissolved

it is easy to secure the blood extract in 15 to 20 c c of chloroform in the flask. Filter the contents of the Soxhlet flask into a 25 c c volumetric flask. Wash out the Soxhlet flask with three or four portions of 1 to 2 c c of chloroform, passing the washings through the filter. Cool the contents of the volumetric flask (if necessary) and add chloroform exactly to the 25 c c mark.

In one glass-stoppered test tube place 5 c c of the "unknown" chloroform extract. In another, place 5 c c of the standard cholesterol solution (8 mgm per 100 c c). To each tube add 2 c c of A R acetic anhydride, and 0.1 c c of concentrated sulphuric acid. Stopper the tubes and mix the contents of each thoroughly. Place both tubes in a basin of water at 22° C for exactly fifteen minutes in a dark cupboard. Then without delay remove the tubes, and compare the colours of the "unknown" and standard in a colorimeter, the cups of which must have their bases fused on (Kober colorimeter) or cemented with chloroform insoluble material.

### Reagents

(1) *Stock Cholesterol* (0.1 per cent) Dissolve 0.1 gm of the purest anhydrous cholesterol<sup>1</sup> in 10 to 20 c c of chloroform in a 100 c c volumetric flask, and add chloroform to the mark.

(2) *Standard Cholesterol Solution* (8 mgm per 100 c c) Pipette accurately 4 c c of the stock cholesterol solution (100 mgm per 100 c c) into a 50 c c volumetric flask, and add chloroform to the mark.

The stock chloroform solution will keep for months in a well stoppered (glass stopper) bottle in the dark. The standard solution usually keeps for a few weeks if similarly well stoppered.

All the chemicals (chloroform, cholesterol acetic anhydride and sulphuric acid) should be of the best quality (A R).

**Calculation** Let  $S$  be the reading of the standard solution,  $U$  of the unknown. Let  $x$  be the quantity of cholesterol in 5 c c of the chloroform extract of blood.

100 c c of the cholesterol standard contain 8 mgm of cholesterol  
Therefore 5 c c contain 0.4 mgm

Therefore  $x \times U = S \times 0.4$ ,

$$\text{or } x = \frac{S}{U} \times 0.4 \text{ mgm}$$

25 c c of the cholesterol extract correspond to 1 c c of blood

5 " " " " " " 0.2 " "

Therefore in 0.2 c c of blood there are  $\frac{S}{U} \times 0.4$  mgm of cholesterol

" 100 " " " "  $\frac{S}{U} \times 0.4 \times \frac{100}{0.2}$  mgm

of cholesterol

<sup>1</sup> Cholesterol in the form of notched plates (crystallised from 90 per cent alcohol) is the monohydrate  $C_{27}H_{48}O \cdot H_2O$  (MW 386 + 18 = 404). Use 0.1047 gm of this instead of 0.1 gm.



Blood cholesterol equals  $\frac{S}{U} \times 200$  mgm per 100 c c

**Notes** Myers and Wardell use a special extraction apparatus but a small Soxhlet apparatus is equally satisfactory. Alternatively the thimble may be suspended in the upper part of a flask containing chloroform by a piece of copper wire fixed into the cork through which passes a reflux condenser.

They propose an alternative standard solution which is an artificial standard consisting of aqueous 0.005 per cent naphthol green B. They find that when this solution is set at 15.5 mm 0.4 mgm of cholesterol in 5 c c of chloroform treated with 2 c c of acetic anhydride and 0.1 c c of concentrated  $H_2SO_4$  will read 15 mm. If a naphthol green B standard is employed the colour must be checked against the treated cholesterol standard.

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*Xanthomatosis* (see Chapter XXVI)

## NON-PROTEIN NITROGEN NITROGEN PARTITION

The nitrogen of the proteins of the blood constitutes about 98 to 99 per cent of the total nitrogen. The remaining 1 or 2 per cent is termed 'non protein nitrogen,' or N P N, because it is nitrogen not contained in protein at the time of the analysis<sup>1</sup>—the term does not refer to the source of the nitrogen. The N P N includes the nitrogen of urea, uric acid, creatinine, creatine, ammonia, amino acids, and the nitrogen of other nitrogenous bodies which is usually labelled "undetermined" or "rest nitrogen." Urea nitrogen accounts for roughly half (33 to 65 per cent) of the non protein nitrogen of normal blood.

Hammett (*J Biol Chem*, 1920 41, 611) studied the nitrogen partition of the whole blood of sixty normal individuals three and a half hours after a meal, with the following results

Substance	Mgm per 100 c c			As Percentage of N P N		
	Highest	Lowest	Average	Highest	Lowest	Average
Non protein N	45.5	27.3	35.6	—	—	—
Urea N	25.1	9.7	17.1	65.7	33.0	47.8
Creatinine N .	0.60	0.37	0.47	1.7	0.9	1.3
Creatine N .	1.78	0.63	1.30	4.9	2.2	3.7
Uric acid N. .	1.16	0.50	0.78	3.6	1.4	2.2
Amino acid N	7.2	3.1	4.9	21.9	8.8	13.8
Rest N. .	18.3	3.7	11.0	48.6	10.4	31.1

The ammonia nitrogen of whole blood is so small (0.1 to 0.2 mgm) that it can be disregarded. It will be noted that the range of variation in the above table is not so wide as that in the table on p. 333, which is compiled from a much larger number of results obtained by many different investigators. It will be seen that in round numbers urea nitrogen constitutes 48, creatinine nitrogen 1, creatine nitrogen 4, uric acid nitrogen 2, amino acid nitrogen 14, and rest nitrogen 31 per cent of the total non protein nitrogen. This should be contrasted with the nitrogen partition of urine.

<sup>1</sup> Strictly speaking the N P N is the nitrogen in the filtrate obtained after precipitating proteins; it therefore does not include lipid nitrogen because the lipids are carried down with the proteins when tungstic or trichloroacetic acid is used as precipitant.

(p 295) urea nitrogen 85, creatinine nitrogen 5, uric acid nitrogen about 15, ammonia nitrogen about 3, and all the remaining nitrogenous constituents (of which amino acids constitute a large part) about 55 per cent of the total (non protein) nitrogen

In diseases accompanied by increase of the blood non protein nitrogen, the percentage which is urea nitrogen increases (cf table on p 99) at the expense of the rest nitrogen, the absolute amount of which is practically unaltered. This is illustrated also in the following table, which, with modifications, is taken from Myers' book

*Nitrogen Partition of Whole blood in per cent of N.P.N*

Condition.	Urea N	Creatinine N	Uric acid N	Remaining N <sup>1</sup>
Normal . . .	48	1	2	49
Gout . . .	48	1	6	45
Parenchymatous nephritis	55	1	2	42
Interstitial nephritis	60 to 70	1 to 2	2	37 to 26
Terminal interstitial nephritis	70 to 80	2 to 2.5	2 to 3	26 to 15

<sup>1</sup> Remaining N here includes creatine nitrogen amino acid nitrogen, and rest nitrogen

From this table it is clear that in health, and still more in disease with nitrogen retention, an estimation of the urea nitrogen gives practically as much information as an estimation of the non protein nitrogen (cf also Chapter V). For this reason the reader is referred to the discussions of blood urea in Chapter V, and later in this chapter, from which he can infer the alterations of non protein nitrogen which may occur in disease.

Though all are agreed that alterations in the blood urea generally reflect the changes of non protein nitrogen, some workers prefer to estimate the latter. For this reason the technique is given below.

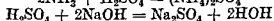
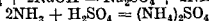
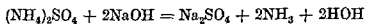
### Technical

The proteins are precipitated by tungstic acid or trichloroacetic acid, and the nitrogen in an aliquot part of the protein free filtrate is determined by a macro Kjeldahl or micro Kjeldahl method, or by digestion and Nesslerisation. For a description of the many modifications of the Kjeldahl procedure the reader is referred to Peters and Van Slyke's *Quantitative Clinical Chemistry*, Vol II.

### Estimation of Non-protein Nitrogen by a Micro-Kjeldahl Method

**Principle** The proteins of whole blood, serum or plasma are precipitated by tungstic acid (see p 332). The nitrogenous bodies of an aliquot part of the protein free filtrate are digested with sulphuric acid to yield ammonium sulphate. (The boiling point of the acid is raised by the addition of the potassium sulphate and the

copper sulphate catalyses the reaction <sup>1</sup>) The ammonia is liberated by adding an excess of caustic soda solution, and is removed by steam distillation into an excess of standard acid. The excess of standard acid is determined by titration with standard NaOH. The difference corresponds to the standard acid neutralised by the ammonia from the nitrogenous bodies of the protein free filtrate, and from this is calculated the non protein N



The Apparatus (Fig 67) is a modification of Pregl's. It consists essentially of a steam generator and a small Kjeldahl flask on to the neck of which is blown a bulb, through which passes the steam

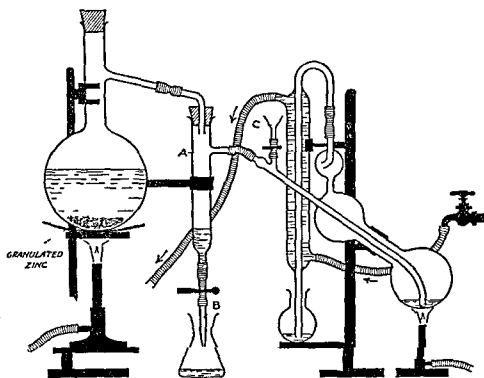


FIG 67 Pregl's micro Kjeldahl apparatus (modified) A, Condensed steam trap B Outlet for condensed steam C Funnel through which solutions are passed into Kjeldahl flask

delivery tube reaching almost to the bottom of the flask, the terminal portion being bent so as to dip well under the fluid in the flask. The steam and liberated ammonia pass up through the bulbous portion and through a trap to a vertical hard glass condenser to which is attached a delivery tube, the tip of which is placed beneath a known volume of standard acid.

The apparatus, apart from the steam generator, condensed

<sup>1</sup> The digestion mixture used for plasma proteins (p 371) may be substituted.

steam trap and condenser, is blown in one piece. Condensed steam is trapped in A. The NaOH is admitted through C. After completing a test the flame is removed from under the steam generator, when, owing to cooling, the contents of the Kjeldahl flask are sucked back into A, and are run off through B before the next test.

It is essential that the whole Kjeldahl apparatus, together with the central and delivery tubes of the condenser, shall all be made of hard glass. If soft glass is employed a large and fluctuating blank will be obtained, owing to solution of varying amounts of alkali from the glass, and the results will be valueless. When a new apparatus has been assembled, steam should be passed through it for four or five hours before undertaking any analyses, after which a low and constant blank will generally result.

**Estimation** Mix in a boiling tube, and then filter

{ Water, ammonia free	16 c c
{ Serum or plasma	2 "
{ 10 per cent sodium tungstate	1 "
{ 2/3 N sulphuric acid	. 1 "

(For whole blood the proportions are 14, 2, 2 and 2 c c respectively)

In a silica or pyrex boiling tube (8 in.  $\times$  1 in.) place

{ 10 c c of the proteïn free filtrate
{ 2 c c of concentrated sulphuric acid, <sup>1</sup> M A R, nitrogen free
{ A pinch of pure potassium sulphate, A R
{ A crystal of pure copper sulphate, A R
{ A glass bead

Mix well and digest over a small flame in a fume cupboard till the mixture is clear—about thirty minutes—and continue the digestion for half an hour after the clearing.

[Whilst the digestion is proceeding, clean the apparatus by passing steam for about half an hour, remove flame and run off trapped water through B, then perform the blank test—see below.]

Allow the digest to cool, add about 4 c c of ammonia free distilled water, mix and pass into the apparatus through C, wash out the digestion tube with a further two portions of water each of about 3 c c, which also pass through C.

Place the 0.01 N sulphuric acid in the conical flask—15 c c is usually ample, *cf* Note—and add a few drops of methyl red solution (*e.g.*, 0.02 to 0.05 per cent in 50 per cent alcohol) as indicator. Put the conical flask in position, with the tip of the delivery tube under the acid.

See that steam is being generated from the boiler. Add through C 10 c c of 40 per cent caustic soda.

Steam distil for at least ten minutes. Lower the receiver, wash the tip of the delivery tube with distilled water, and continue the

<sup>1</sup> The digestion mixture used for plasma proteins (p. 371) may be substituted

distillation for another two minutes to wash the inside of the delivery tube. Titrate the distillate with 0.01 N caustic soda.

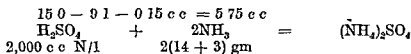
A Blank test must be performed by substituting water for the blood or plasma. Thus in a blank for the estimation in plasma, mix 18 c.c. of water, 1 c.c. of 10 per cent tungstate and 1 c.c. of 2/3 N acid, and filter through the same grade of filter paper as used in the test proper. Take 10 c.c. of the filtrate and carry out the digestion and subsequent steps exactly as in the test itself. The blank value should not exceed 15-14.75 or 0.25 c.c. of 0.01 N alkali.

**Example of Calculation** 15 c.c. of 0.01 N sulphuric acid were placed in the receiver.

The titration was 9.1 c.c. of 0.01 N NaOH.

The blank was 0.15 c.c. of 0.01 N alkali.

Therefore, the acid neutralised by the ammonia from the protein-free filtrate was



Since 1,000 c.c. of N/1  $\text{H}_2\text{SO}_4$  correspond to 17 gm. of ammonia, or 14 gm. of ammonia nitrogen,

$$\begin{array}{rcl}
 1 \text{ c.c. of N/1 H}_2\text{SO}_4 & = & 14 \text{ mgm. of N} \\
 1 \text{ c.c. of N/100 H}_2\text{SO}_4 & = & 0.14 \text{ mgm. of N} \\
 5.75 \text{ c.c. of N/100 H}_2\text{SO}_4 & = & 0.14 \times 5.75 \text{ mgm. of N} \\
 & = & 0.805 \text{ mgm. of N}
 \end{array}$$

Since 10 c.c. of protein free filtrate = 1 c.c. of blood, plasma or serum,

$$\begin{array}{rcl}
 1 \text{ c.c. contains} & 0.805 \text{ mgm. of N} \\
 100 \text{ c.c. contain} & 80.5 \text{ mgm. of N} \\
 \text{i.e.,} \quad \text{NPN} & = & 80.5 \text{ mgm. per 100 c.c.}
 \end{array}$$

**Note** When the NPN is very high (over about 180 mgm.), use more (e.g., 25 c.c.) of the 0.01 N acid, or less filtrate (e.g., 5 c.c.)

## OXYGEN

Oxygen is carried in the blood in loose combination with hæmoglobin, though there is also a minute trace in physical solution. The quantity of oxygen in the blood, therefore, depends almost entirely on the concentration of hæmoglobin.

By the oxygen capacity is implied the number of cubic centimetres of oxygen which 100 c.c. of whole blood fully saturated with oxygen (air) can carry. It may conveniently be determined with the aid of the Van Slyke apparatus (p. 198). For a detailed description of the method the reader is referred to Peters and Van Slyke's book (Vol. II). In brief, a known volume of blood is saturated with air in the apparatus, and then laked with air free water. Potassium ferricyanide solution is added and the apparatus is evacuated. The evolved gas is a mixture of oxygen, nitrogen and carbon dioxide. The  $\text{CO}_2$  is absorbed with  $\text{NaOH}$  solution, and the volume of the residual  $\text{O}_2 + \text{N}_2$  is measured. Corrections are made for temperature, barometric pressure, aqueous vapour pressure and nitrogen. The oxygen capacity includes the oxygen combined with hæmoglobin and the oxygen in physical solution.

The oxygen combining power is the cubic centimetres of oxygen combined with the hæmoglobin in 100 c.c. of blood fully saturated with oxygen (air). It is the oxygen capacity minus the oxygen in physical solution.

The hæmoglobin concentration may be calculated from the oxygen capacity after making allowance for the oxygen in physical solution, i.e., from the oxygen combining power. One c.c. of oxygen combines with 0.746 gm. of hæmoglobin.

The oxygen content is the number of cubic centimetres of oxygen (at N.T.P.) in 100 c.c. of blood, the blood being obtained without loss of gases and without admission of air (cf. Chapter XVII). Analyses may be made on arterial and on venous blood, analyses of both are usually necessary in assessing the type and degree of anoxæmia. With but slight differences, the technique of the determination of the oxygen content is the same as that of the oxygen capacity.

The oxygen saturation (of hæmoglobin) is the quantity of oxygen combined with the hæmoglobin in 100 c.c. of blood, expressed as a percentage of the oxygen combining power of that particular blood. It is therefore the "oxygen content minus  $\text{O}_2$  in physical solution," expressed as a percentage of the "oxygen capacity minus  $\text{O}_2$  in physical solution," or

$$\frac{(\text{c.c. } \text{O}_2 \text{ content} - 0.2 \text{ c.c.}^1)}{(\text{c.c. } \text{O}_2 \text{ capacity} - 0.5 \text{ c.c.})} \times 100$$

<sup>1</sup> Arterial blood see table on p. 359

The oxygen unsaturation is equal to 100 — oxygen saturation. It is often calculated as the oxygen capacity less the oxygen content, though strictly this is not correct, because of the oxygen capacity value about 0.5 c.c. is in physical solution whereas of the oxygen content value about 0.1 c.c. (venous) or 0.2 c.c. (arterial) is in physical solution (see table below).

The oxygen tension or oxygen pressure of the blood is equal to the pressure of the oxygen in an atmosphere with which that blood is in equilibrium. It cannot be measured directly by simply extracting all the gases from the blood and determining the composition of the extracted gas mixture, because the oxygen (and  $\text{CO}_2$ ) is present not only in physical solution, but also in chemical combination. It must, therefore, be determined indirectly with the aid of an "oxygen dissociation curve" or a "hæmoglobin dissociation curve" (cf  $\text{CO}_2$  tension, Chapter IX).

The different oxygen values obtained in healthy males are given in the following table which is compiled mainly from the publications of Stadie, Harrop, Gram, and Wright.

*Oxygen Values in Healthy Males (Whole Blood) (Cubic Centimetres per 100 c.c. of Blood, unless otherwise stated)*

	Max	Min.	Average
Oxygen capacity ( $\text{O}_2$ in combination plus $\text{O}_2$ in solution)	24.7	14.4	20.4
Oxygen combining power ( $\text{O}_2$ in combination) <sup>1</sup>	22.2	17.8	20.0
Oxygen in physical solution (blood saturated with $\text{O}_2$ )	—	—	0.5
Arterial oxygen content	24.1	13.9	19.6
(Oxygen in physical solution)	—	—	0.2
Venous oxygen content	18.0	9.6	14.1
(Oxygen in physical solution)	—	—	0.1
Arterial oxygen unsaturation <sup>2</sup>			
Cubic centimetres per 100 c.c.	1.4	0.6	1.0
Per cent of oxygen combining power	6.5	2.8	5.0
Venous oxygen unsaturation			
Cubic centimetres per 100 c.c.	6.3	4.9	5.6
Per cent of oxygen combining power	33.0	22.7	26.6
Arterial oxygen tension millimetres	100.0	84.0	—
Venous oxygen tension millimetres	60.0	30.0	—

<sup>1</sup> Corresponding figures for oxygen combining power of healthy females' blood are max 18.9 min 16.1 and av 17.6 c.c. per 100 c.c. of blood. These are lower than the values for males because the blood of healthy women contains a lower concentration of hæmoglobin.

<sup>2</sup> The oxygen saturation = 100 — oxygen unsaturation (expressed as per cent of oxygen combining power).

When the tissues receive an inadequate supply of oxygen anoxæmia is present. Barcroft has classified anoxæmia in three groups, anoxic (arterial), stagnant and anæmic. In the anoxic type the arterial oxygen unsaturation exceeds 6 per cent, or, in other words, the hæmoglobin is less than 94 per cent saturated with



oxygen The anoxæmia may be due to diminished partial pressure of oxygen in the atmosphere (at high altitudes in flying or in mountaineering) or to pulmonary disease (pneumonia, cedema, emphysema) The shallow rapid breathing is probably the cause of the anoxæmia in lung diseases, some of the lobules are incompletely expanded, with the result that the blood passing through these is not fully saturated In lobar pneumonia the blood supply appears to be diverted from the consolidated area, so that the anoxæmia must be explained by the uneven ventilation of the non consolidated areas (*cf* Haldane)

In the stagnant type the arterial oxygen saturation is normal (*cf* Fraser), but impaired circulation results in an increased abstraction of oxygen from the blood by the tissues, so that the venous oxygen saturation falls below the normal lower limit of 67 per cent This occurs in uncompensated heart diseases, and locally (*e g*, in the extremities), due to cold, Raynaud's disease, etc

In the anæmic type the quantity of available hæmoglobin is diminished, either due to anæmia (percentage of hæmoglobin lowered) or to fixation of some of the hæmoglobin, as in CO poisoning, methæmoglobinæmia, sulphæmoglobinæmia, etc (percentage of *available* hæmoglobin reduced) In anæmia the percentage oxygen saturation of hæmoglobin is normal or above normal but the oxygen capacity and oxygen content of the blood are reduced in comparison with the values in health In CO poisoning etc the percentage oxygen saturation of the total hæmoglobin is reduced

Oxygen determinations have thrown considerable light on the significance of cyanosis (see Lundsgaard and van Slyke) It has been shown that cyanosis does not occur until about 5 gm of hæmoglobin per 100 c c of *capillary* blood are in the reduced form (5 gm per cent of reduced hæmoglobin corresponds to an oxygen unsaturation of about 6.7 c c per 100 c c of blood), or in the reduced plus an unavailable form (*cf* methæmoglobinæmia and sulphæmoglobinæmia, Chapter XVIII) Anæmic patients do not readily become cyanosed and when the hæmoglobin falls below about 30 per cent of the normal, cyanosis is impossible, since the blood contains less than the necessary 5 gm of hæmoglobin per 100 c c On the other hand, in polycythæmia the hæmoglobin percentage is so much above normal that 5 gm of hæmoglobin per 100 c c are commonly in the reduced form, and therefore there is cyanosis, although the tissues are well supplied with oxygen In other words, in order to interpret the significance of cyanosis (or its absence), the hæmoglobin percentage, or rather the available hæmoglobin percentage (oxygen combining power), should be known As already stated, cyanosis is dependent on the quantity of capillary hæmoglobin which is reduced and/or altered to CO hæmoglobin, etc Cyanosis may, therefore, occur even when the arterial oxygen saturation is normal, provided that the capillary oxygen saturation is low enough In other words, when studying cyanosis, determinations of oxygen saturation should be made on venous blood

(obtained without constriction and without loss of blood gases or admission of air), and on the arterial blood, in order to decide whether or not the cyanosis is due to local circulatory conditions

### Clinical Value

Although oxygen determinations in the blood have been of the greatest assistance in extending our knowledge of the causes of the symptoms in pulmonary, cardiac and blood diseases, and in defining the limitations of oxygen therapy, they are of only limited value in practical medicine. The clinical worker can usually obtain all the information he requires from his bedside observations and blood examinations (hæmoglobin determinations, blood counts and spectroscopical examination for abnormal pigments). Oxygen determinations are valuable, however, for putting his observations on a quantitative basis. Thus most interesting results have been obtained in pneumonia by Stadie, but such rapid changes may occur in this disease, and the opportunity for chemical tests must of necessity be limited by the severity of the illness, that the clinical observer is not materially assisted in prognosis or treatment.

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## PHOSPHATASE

Phosphatases, enzymes which hydrolyse phosphate esters to liberate inorganic phosphate, are widely distributed in mammalian tissues, being in greatest concentration in bone, kidney, intestinal mucosa and liver. They are present also in red cells, which makes it essential to take special precautions in determining the inorganic phosphate of plasma (see next section on Phosphorus). Traces are found also in plasma or serum which has been completely freed of cells. The origin of this phosphatase of normal plasma or serum is still a disputed point, there may be a continual leakage of traces of the enzyme from the bones, or from one or more of the other phosphatase containing tissues, or from all such tissues.

Pathologically it has been clearly shown that the plasma phosphatase is increased in active bone diseases (Kay) and in obstructive jaundice (Roberts). The clinical application of these observations, however, has on the whole been disappointing, involvement of bone is generally obvious on other grounds, and attempts to distinguish between different types of jaundice have met with only limited success because there is considerable overlapping of results in the different groups.

The determination of plasma phosphatase depends upon the liberation of extra inorganic phosphate by the enzyme from sodium  $\beta$  glycerophosphate solution added to the plasma. The initial inorganic phosphate, and the total inorganic phosphate at the end of a given period of incubation, are estimated, the difference is a measure of the quantity of the ferment. The incubation in Kay's original method took forty eight hours, this was modified by Roberts to two hours (whole blood used), and by Jenner and Kay to three hours. The phosphorus is estimated by different techniques, and the normal values are different in the three methods, it is therefore essential to name the technique when recording results.

Method*	Definitions* of Unit	Normal Range Units
Kay	1 mgm. P liberated by 1 c.c. plasma in forty eight hours	{ 31 adults 0.08 to 0.21 8 infants 0.17 to 0.34 10 adults, 3.2 to 7.9
Jenner and Kay	1 mgm. P liberated by 100 c.c. plasma in three hours	
Roberts	1 mgm. P liberated by 100 c.c. whole blood in two hours	Up to 5.5

\* The definitions here are incomplete—see original papers for full details—but are sufficient to show that the units of enzyme and therefore the normal ranges must be different by the three methods.

Kay's results in pathological conditions may be summarised briefly as follows —

31 Healthy adults	0.08 to 0.21 units
Over 150 miscellaneous pathological, not bony lesions	0.1 „ 0.5 „ (majority 0.1 to 0.3)
10 Hyperthyroidism	Many, but not all, show slight to moderate rise seldom exceeding 0.7 units
9 Osteomyelitis	
6 Fragilitas ossium	
28 Fractures and tumours of bone	Almost all show a well marked rise of above 0.5 to over 3 units
13 Rickets	
8 Osteomalacia	
34 Osteitis deformans	
10 Generalised osteitis fibrosa	

To these may be added Anderson's findings in jaundice, using the same method —

11 Obstructive jaundice	0.45 to 1.44 units
12 Toxic and infective jaundice	0.15 „ 0.74 „
2 Haemolytic jaundice	0.14 „ 0.18 „

Herbert's analyses in jaundice show similar overlapping, she used Jenner and Kay's method —

10 Healthy adults <sup>1</sup>	3.2 to 7.9 units
20 Miscellaneous diseases (not bony, not biliary) <sup>1</sup>	4.5 „ 14.3 „
28 Obstructive jaundice	12.9 „ 120 „
24 Toxic and infective jaundice	2.8 „ 33.4 „
14 Haemolytic jaundice	2.6 „ 13.4 „

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<sup>1</sup> Jenner and Kay's findings

## PHOSPHORUS

A good review of the physiology of phosphorus is given in Peters and Van Slyke's book. An intensive study of blood phosphorus has been undertaken by Kay and Byrom, who classify the different types of phosphorus compounds found in whole blood as follows —

- |   |  |  |
|---|--|--|
| (1) Inorganic phosphate                 | $\left. \begin{array}{l} \text{Ester or} \\ \text{"organic",} \\ \text{phosphorus} \end{array} \right\}$           | $\left. \begin{array}{l} \text{"Acid-} \\ \text{soluble} \\ \text{phosphorus"} \end{array} \right\}$ |
| (2) Nucleotide phosphate                |  |  |
| (3) Phosphoglyceric acid                |  |  |
| (4) Reducing phosphoric ester           |  |  |
| (5) At least one other phosphoric ester |  |  |
| (6) Lecithin                            | $\left. \begin{array}{l} \\ \\ \end{array} \right\}$   | Lipin phosphorus (phosphatides)  |
| (7) Kephalin                            |  |  |
| (8) Sphingomyelin                       |  |  |
| (9) Nucleic acid                        | $\left\{ \begin{array}{l} \text{Residual phosphorus (very small in total} \\ \text{quantity)} \end{array} \right.$ |  |

Four analyses are required, viz., inorganic phosphate, acid soluble phosphorus, lipin phosphorus, and total phosphorus. Alternatively the first three may be made, and the total phosphorus may be calculated by adding together the acid soluble and lipin phosphorus without appreciable error. The ester phosphorus is calculated as acid soluble minus inorganic phosphorus. The normal values for these fractions are given on p. 335. Except for inorganic phosphate (see end of this section), the reader is referred to the articles quoted for the methods of estimating the different fractions.

Apart from the inorganic phosphate fraction, comparatively little of direct practical clinical value has so far emerged, though the observations are of the greatest interest to physiologists and pathologists. Further description here will, therefore, be limited to the inorganic phosphate of the blood.

## Inorganic Phosphate

Estimations are generally made on plasma, which has been separated as soon as possible after withdrawal of the blood, in order to avoid the formation of extra inorganic phosphate by enzyme hydrolysis of the ester phosphorus in the corpuscles (see under Technical).

The inorganic phosphate of the plasma is higher in infants and children than in adults (see p. 335), the difference probably being due to the active ossification of the child. The inorganic phosphate falls slightly in pregnancy, presumably owing to the demands of foetal ossification. A slight rise occurs during lactation, and is probably connected with the formation of milk. In infants (and

also in adults), there appears to be a seasonal tide of blood inorganic phosphate, which may be of significance in the seasonal incidence of rickets

*Inorganic P Decreased*

Active rickets  
Osteomalacia (some cases)  
Generalised osteitis fibrosa  
(hyperparathyroidism)  
By insulin

*Inorganic P Increased*

Chronic nephritis  
Diabetic coma  
Renal infantilism (some cases)  
During healing of major fractures

The chief value of inorganic phosphate estimations is in rickets. They are of distinct value in diagnosis, and particularly in the early stages of the disease, though it must be remembered that low values are not pathognomonic of the disease. In round numbers the serum or plasma inorganic phosphate of healthy children lies between 4 and 6 mgm per 100 c c, though the reported normal figures cover a slightly wider range viz, 3.2 to 6.5 mgm. In rickets the serum inorganic phosphate may fall below 1 mgm and is commonly below 3 mgm. Thus Howland and Kramer in their series, found values of 0.6 to 3.2 (average 2.0 mgm). The phosphorus estimation is more valuable in this disease than a determination of serum calcium, since the low phosphate type of rickets is commoner than the low calcium type. Kramer, Tisdall and Howland find the product of the two ( $\text{Ca} \times \text{P}$ ) useful in the diagnosis of rickets. In health this product exceeds 40.

Wills found no significant lowering of the plasma inorganic phosphate of a series of children with marked lack of muscle tone, but no evidence of bony rickets. Her results, which are well-controlled, are given below.

Type of Case	Age Years	No of Cases	Mgm. Per 100 c c Plasma		
			Lowest	Highest	Average
Normal . . .	5 to 10	5	4.1	6.1	4.8
Inflamed tonsils and adenoids but good muscle tone . .	3 to 12	20	2.9	5.1	4.1
Poor muscle tone .	1 to 11	25	2.5	6.1	4.5

The rise in the serum or plasma inorganic phosphate and its prognostic significance in chronic nephritis have already been discussed in Chapter V. The raised values in diabetic coma are probably the result of renal injury.

In renal infantilism a rise in the inorganic phosphate of the plasma or serum sometimes occurs (*cf* Parsons), and may be ascribed

to the kidney disease, the blood chemistry being very similar to that found in advanced chronic interstitial nephritis

In healthy adults the inorganic P per 100 c.c. of plasma or serum lies between 2 and 4 mgm. Following major fractures it may rise slightly (to about 6 mgm.), and slowly during the healing of the bone, whereas cases of non union do not usually show this reaction

Injections of insulin cause a fall in the inorganic phosphate of the blood. Phosphoric esters are synthesised in the blood corpuscles at the expense of the inorganic phosphate. It is probable also that an intermediate compound of phosphate and carbohydrate is formed in the muscles in the utilisation of glucose subsequent to glucose or insulin administration. The fall in the inorganic phosphate is brought about by a redistribution of phosphorus, and not by a loss from the body

### Estimation of Inorganic Phosphate

Many methods have been introduced for the estimation of the inorganic phosphate of blood, which are critically discussed in Peters and Van Slyke's *Quantitative Clinical Chemistry*, Vol. II. Briggs' method is selected for description here, because it is simple and has perhaps been more widely employed than any other in clinical work

**Collection of Blood and Preparation for Analysis** The blood is collected from a vein in the usual way (Chapter XVII). The corpuscles contain "ester phosphorus" compounds which rapidly hydrolyse in shed blood, liberating extra inorganic phosphate. To avoid this artificial increase of inorganic phosphate it is essential either to separate the corpuscles as soon as possible by centrifuging (for plasma analyses), or to carry out the treatment of the whole blood without delay. Since the plasma contains only small quantities of hydrolysable ester phosphorus, once the plasma is separated delay is of little or no consequence. Analyses are therefore nearly always performed on plasma (separated within half an hour) or on serum (separated within two hours). For the reason given, it is also obviously necessary to avoid hæmolysis. Plasma showing a definite red tinge of hæmoglobin should be discarded.

If the analysis has to be made on whole blood, a measured volume should be laked at once with the requisite amount of water, and precipitated with the trichloroacetic acid without delay. But the same hydrolysis will occur in the acid protein free filtrate of whole blood if it is kept, so that it is necessary to proceed with the analysis at once.

Burkens has shown that this liberation of extra inorganic phosphate from ester phosphorus can be prevented by using as anticoagulant 0.2 gm. of NaF per 100 c.c. (poisonous action on phosphatase). This would be very valuable for whole blood, but is not essential for plasma (or serum) provided it can be separated rapidly as described above, for, as already mentioned, the ester P content of plasma is slight.

**Principle** The proteins in a measured volume of blood are precipitated with trichloroacetic acid. An aliquot part of the protein free filtrate is treated with molybdic acid, and the resulting phosphomolybdate is reduced by hydroquinone in the presence of acid and of sodium sulphite. The reduction compound is blue and non fading under these conditions. Its colour is compared with that of a standard phosphate solution similarly treated.

### Reagents

(a) *Molybdic Acid Solution* Dissolve 25 gm of pure ammonium molybdate in 300 c c of distilled water. Add cautiously 75 c c of sulphuric acid to about 100 c c of distilled water in a measuring cylinder. Cool, add to the ammonium molybdate solution, and make up to 500 c c.

(b) *Hydroquinone Solution* Dissolve 0.5 gm of hydroquinone in distilled water and make up to 100 c c, add 1 drop of concentrated sulphuric acid to retard oxidation. It keeps for a few days.

(c) *Sodium Sulphite Solution* Dissolve 20 gm of crystalline sodium sulphite  $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$  in distilled water to 100 c c and stopper well. The solution is stable for only a few weeks.

(d) *Standard Phosphate Solution* Dissolve 0.0878 gm of pure dry acid potassium phosphate ( $\text{KH}_2\text{PO}_4$ ) in distilled water to 1,000 c c. Add chloroform as preservative. Two c c of this solution is equivalent to 0.04 mgm of phosphorus.

(e) *Twenty per cent Trichloroacetic Acid*

**Technique** In a test-tube place

- { 2 c c of plasma or serum (or whole blood),
- { 6 c c of distilled water,
- { 2 c c of 20 per cent trichloroacetic acid

Close the mouth of the tube with the thumb, and shake vigorously for a few seconds.

Allow the mixture to stand for ten minutes, and then filter through a double acid washed filter paper.

In a glass stoppered test tube graduated at 10 c c place

- { 5 c c of the protein free filtrate,
- { 2 c c of the molybdic acid solution,
- { 1 c c of the sodium sulphite solution,
- { 1 c c of the hydroquinone solution,

and add water to the 10 c c mark.

In another similar tube, place

- { 2 c c of the phosphate standard (= 0.04 mgm. P),
- { 2 c c of the molybdic acid solution,
- { 1 c c of the sodium sulphite solution,
- { 1 c c of the hydroquinone solution,

and add water to the 10 c c mark.

Standard and unknown should be prepared simultaneously



Each is stoppered, inverted to mix, and allowed to stand for thirty minutes before comparison in the colorimeter

**Calculation** Let  $S$  be the reading of the standard, and  $U$  of the unknown. Let  $x$  be the mgm of phosphorus in the 5 c c of plasma filtrate used

Since the standard contains 0.04 mgm of P,

$$x \times U = S \times 0.04,$$

$$\therefore x = \frac{S}{U} \times 0.04$$

Since 5 c c of filtrate correspond to 1 c c of plasma,

$$1 \text{ c c of plasma contains } \frac{S}{U} \times 0.04 \text{ mgm of P}$$

$$100 \text{ ,, ,, contain } \frac{S}{U} \times 4.0 \text{ mgm of P}$$

If the plasma contains much more than 4.0 mgm of P per 100 c c, a greater amount of the standard phosphate solution (or less of the plasma filtrate) should be used, to insure accurate colorimetric determinations

**Notes** (1) Briggs found that reasonable quantities (cf Chapter XVII) of anticoagulant (oxalate or citrate) did not interfere with the development of the colour

(2) Briggs states that it is not necessary to add trichloroacetic acid to the standard to balance that in the unknown. It is, however, necessary that the acidity of the standard shall be within certain limits. This he has accomplished by the acidity produced by the added molybdic acid solution. If the acidity is too high (more than 2 N in the final mixture) the blue colour is not developed

(3) In a modification of the above method aminonaphthol sulphonic acid is used as reducing agent instead of hydroquinone—Fiske and Subarrow, *J Biol Chem*, 1925, 66, 375 (See also Hawk and Bergeim's *Practical Physiological Chemistry*)

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### THE PROTEINS OF PLASMA OR SERUM

The proteins of plasma are albumin, globulins, and fibrinogen. "Total protein" is the sum of the three. The normal values for the total and the fractions are given on p. 336. Though it must be admitted that the differential estimation of proteins is more of academic interest than of practical clinical value, requests are sufficiently numerous to necessitate the inclusion of the technique.

Fibrin is separated by recalcifying oxalated or citrated plasma. Globulins (and fibrinogen) are precipitated by saturation with magnesium sulphate, or by half saturation with ammonium sulphate (except when nitrogen estimations follow the fractionation) or by 22 per cent sodium sulphate,<sup>1</sup> and the albumin is determined in the filtrate. In another portion of the fibrin free plasma, the albumin plus globulin is determined. Globulin is calculated by difference. When fibrin(ogen) estimations are not required, serum may conveniently be employed for the estimation of albumin and of globulins, and serum is often preferred in routine clinical work.

The total protein, or the protein in each fraction, may be estimated refractometrically, colorimetrically, or with the aid of nitrogen (Kjeldahl) determinations. The refractometric results are criticised severely by Peters and Van Slyke (see their book) because they are often higher than the Kjeldahl and colorimetric findings, and especially when the plasma contains much lipid.

### Pathological Findings

The blood fibrin has already been discussed in Chapter XII, in connection with liver disease. Low values are found in severe degeneration of the liver, but unfortunately so many factors tend to raise the fibrin percentage that the test is of little value in hepatic diseases. Raised values are encountered in a great many diseases (most infections, cancer, nephritis, pregnancy, etc.), and the estimation is of little or no value clinically.

The total protein is increased by gross dehydration, as in cholera and some other conditions with intense diarrhoea, or in very severe and repeated vomiting; this is simply a concentration owing to loss of water, and affects albumin and globulins proportionately. The total proteins are increased also in some cases of kala azar (up to 10 gm per 100 c.c.) and of multiple myelomatosis (up to 16 gm per 100 c.c.), in kala azar the increase is due to globulin (*cf.* Wu), and the possible connection of this with the strongly positive formol gel test has been mentioned in Chapter XVIII, in a proportion, but in by no means all cases of multiple myelomatosis, it is

<sup>1</sup> Fractionation by 22 per cent sodium sulphate (Howe) may give different results from fractionation by half saturation with ammonium sulphate (*cf.* Fine) the albumin being higher when the ammonium salt is used.

again the globulin fraction which is raised, and it has been suggested from evidence which technically is not satisfactory, that Bence Jones' protein itself is responsible in part at any rate for the rise in globulin

The total protein is decreased by loss through the kidneys, with a resulting marked proteinuria, especially in nephrosis, as has been described in Chapter V, and by severe malnutrition (famine œdema, wasting diseases, some cases of diabetes, etc.) In both the renal and nutritional groups the fall is generally confined to the albumin fraction and is often accompanied by œdema (cf Chapter V)

For a fuller discussion the reader is referred to Peters and Van Slyke's *Quantitative Clinical Chemistry*, Interpretations in Vol I and Methods in Vol II The techniques selected for description below are those which have been found most useful in routine clinical work in the writer's laboratory Some form of Kjeldahl determination, which is generally regarded as the standard procedure, is essential in all well equipped laboratories, but a colorimetric method is often preferred in clinical work, Wu's technique (protein, or protein fraction, treated with molybdate tungstate reagent and compared colorimetrically with a tyrosine standard similarly treated) has been employed extensively, but Fine's application of the biuret method is simpler

### Micro-Kjeldahl Method for Plasma or Serum Proteins

The total protein is calculated from the total nitrogen after subtraction of the non protein nitrogen, which is estimated as described on p 354 or is allowed for as explained below, the principle of the nitrogen determinations has also been given on p 354 the only difference being in the digestion mixture used for plasma and serum

In the differential protein estimations globulins are precipitated from serum or globulins plus fibrinogen from plasma, by 22 per cent sodium sulphate the albumin plus N P N is determined in the filtrate Fibrinogen is separated as fibrin by recalcification of plasma The same process of digestion, micro Kjeldahl distillation, titration, and calculation of nitrogen and hence protein, are applied to each fraction It is therefore convenient to divide the description of the method into a series of steps as given below

### Reagents

*Physiological saline*, 0.85 or 0.9 per cent NaCl

*22 per cent Sodium Sulphate* Dissolve 220 gm of anhydrous sodium sulphate (e.g., Kahlbaum's) in about 650 c.c. of water by warming (to about 60° C.) Transfer with the aid of a little warm water to a 1000 c.c. volumetric flask Place in the incubator and make up to the mark at 37° C

*Calcium Chloride Solution* 2.5 gm of anhydrous  $\text{CaCl}_2$  or 5 gm of crystalline  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$  dissolved in water and made up to 100 c.c.

**Digestion Mixture** Mix well 3 parts of concentrated sulphuric acid (M A R, nitrogen free) and 1 part of syrupy phosphoric acid, S G 175

**Potassium Persulphate** The solid salt of analytical quality and substantially ammonia free (B D H AnalaR reagent is suitable)  
40 per cent NaOH

### (1) Separation of Protein Fractions in Serum

For Total Protein (Albumin N + Globulin N + N P N), mix

Serum	0.5 c c
0.85 or 0.9 per cent NaCl	4.5 "

and use 1 c c of the mixture for the digestion (1 c c of mixture  $\equiv$  0.1 c c of serum)

For Albumin (Albumin N + N P N), mix in a stoppered flask,

Serum	0.5 c c
22 per cent sodium sulphate	15 "
Thymol a small crystal	

and place in the incubator at 37° or 38° C for three hours. Filter in the incubator through hardened filter paper (e g, Whatman No 44) until the filtrate is clear. Use 5 c c of the filtrate for the digestion (5 c c of filtrate  $\equiv \frac{5}{31}$  c c of serum)

Globulins are calculated by difference from the two determinations above. Globulin N = (Albumin N + Globulin N + N P N) - (Albumin N + N P N)

For N P N proceed as described on p 354, or allow for as explained below

### (2) Separation of Protein Fractions in Plasma

For Fibrinogen mix in a boiling tube or small beaker

0.85 or 0.9 per cent NaCl	28 c c
Plasma	1 "
Calcium chloride solution	1 "

and place in the incubator at 37° or 38° C for thirty minutes<sup>1</sup>. Insert a capillary pipette with sealed tip or a slender glass rod with a pointed end, and whip gently, the fibrin initially a jelly, contracts enormously and adheres to the rod. Filter, and just before completion of filtration inspect carefully, and if necessary pick up on the rod any bits of fibrin which became detached in the original whipping process. Grip the fibrin in a dry folded filter paper and slip it off the rod. Then press the clot in the paper to absorb adherent fluid as thoroughly as possible. Transfer the clot to the digestion tube, together with about 1 c c of water.

For Albumin + Globulin (Albumin N + Globulin N + N P N)

<sup>1</sup> Occasionally 30 minutes are not enough and the mixture may have to be left for an hour or even overnight for a gel to be formed.

Use 3 c c of the filtrate obtained in the above separation of fibrin, for the digestion (3 c c of filtrate  $\equiv$  0.1 c c plasma)

For Albumin (Albumin N + N P N) treat a mixture of 0.5 c c plasma, 15 c c of  $\text{Na}_2\text{SO}_4$  solution and 1 thymol crystal exactly as described in the previous section for serum

Globulins are calculated by difference Globulin N = (Albumin N + Globulin N + N P N) - (Albumin N + N P N)

For N P N proceed as described on p 354, or allow for as explained below

### (3) The Digestion

The quantity of diluted serum, or of filtrate from serum or plasma, or of fibrin as indicated in sections (1) and (2) above, is placed in a Pyrex tube 8 in  $\times$  1 in. Add 1 c c of the acid digestion mixture, and a glass bead if desired

Heat over a micro burner until charring begins and definite white fumes appear, this often occurs when the volume has been reduced to about one third. But if the volume gets very low before white fumes appear, add about 1 c c of warm water and continue the heating until there are white fumes. Allow the tube to cool considerably and add 1 gm of solid potassium persulphate and 1 c c of water. Heat again until the contents of the tube are quite clear (generally about fifteen minutes). Cool and wash into the micro-Kjeldahl flask with three portions each of about 3 c c of water through inlet C in the figure on p 355

### (4) Micro-Kjeldahl Distillation and Titration

Place 10 c c of 0.01 N  $\text{H}_2\text{SO}_4$  and a few drops of methyl red solution (0.02 to 0.05 per cent in 50 per cent alcohol) in the conical flask receiver which place in position with the tip of the delivery tube under the acid

See that steam is being generated from the boiler. Add, through C, 10 c c of 40 per cent NaOH, and steam distil for at least ten minutes. Lower the receiver, wash the tip of the delivery tube with distilled water, and continue the distillation for another two minutes to wash the inside of the delivery tube. Titrate the distillate with 0.01 N caustic soda

[The acid and alkali may be  $\frac{N}{100}$ ,  $\frac{N}{70}$  or  $\frac{N}{50}$ , as preferred]

### (5) Apparatus and Blanks

The apparatus is a modification of Pregl's and has been fully described on p 355. The methods of preparing it, using it and emptying it have also been described (p 356)

It is advisable to carry out the appropriate blank just before (or just after) each test, water being substituted for serum or plasma, thus 1 c c of a mixture of 0.5 c c water plus 4.5 c c of physiological saline (or 1 c c of the saline) is used in the blank for serum total protein, and 5 c c of 22 per cent  $\text{Na}_2\text{SO}_4$  in the blanks for serum or plasma albumin, and so on. The digestion with the sulphuric-

phosphoric mixture and persulphate must be carried out just as in the test proper. Blank tests are essential, and must never be omitted. They should not exceed 10 — 9.75 or 0.25 c.c. of 0.01 N alkali.

#### (6) Note on Allowance for N.P.N.

Naturally it is best actually to estimate the N.P.N. In routine work, however, when speed is important, an allowance may be made for the N.P.N. without introducing a serious error. If from the clinical examination, including routine urinary tests it is concluded that there is no likelihood of a condition which might cause nitrogenous retention, the average normal N.P.N., viz., 25 mgm. per 100 c.c. of serum or plasma, may be utilised. If the blood urea has been estimated recently, that may be used for computing the N.P.N. as follows —

<i>Blood urea (whole blood plasma or serum)</i>	<i>Factor for converting blood urea into plasma N.P.N.</i>
up to 50	0.9
51 to 100	0.8
101 to 150	0.7
151 to 200	0.6
201 and over	0.55

#### (7) Example of Calculations for Serum

For Total Protein (Albumin N + Globulin N + N.P.N.) The N.P.N. (see p. 354) was 60 mgm. per 100 c.c. The blank was 10 — 9.8 c.c. or 0.2 c.c. of 0.01 N alkali. The equivalent of 0.1 c.c. serum (see p. 371) was employed. 10 c.c. of 0.01 N  $\text{H}_2\text{SO}_4$  was used to catch the ammonia, and 2.6 c.c. of 0.01 N NaOH were required to neutralise the excess acid. Since 1,000 c.c. of 1.0 N  $\text{H}_2\text{SO}_4$  correspond to 14 gm. of ammonia nitrogen,

1 c.c. of 0.01 N  $\text{H}_2\text{SO}_4$  corresponds to 0.14 mgm. of N (cf p. 357)

The acid neutralised by the ammonia from 0.1 c.c. of serum was therefore 10 — 2.6 — 0.2, or 7.2 c.c. 0.01 N.

The nitrogen from 0.1 c.c. of serum was therefore  $7.2 \times 0.14$ , or 1.008 mgm.

100 c.c. of serum therefore contained  $1.008 \times 1,000$  mgm. of N or 1.008 gm. of N. But the N.P.N. accounted for 0.060 gm. of this. The protein N therefore amounted to  $1.008 - 0.060$ , or 0.948 gm. per cent.

The total protein was therefore  $0.948 \times 6.25$ , or 5.93 per cent.

For Albumin (Albumin N + N.P.N.) The N.P.N. was 60 mgm. per 100 c.c. and the blank on 5 c.c. of 22 per cent  $\text{Na}_2\text{SO}_4$  was 10 — 9.8, or 0.2 c.c. of 0.01 N alkali. The equivalent of  $\frac{5}{31}$  c.c. of serum (see p. 371) was employed, 10 c.c. of 0.01 N  $\text{H}_2\text{SO}_4$  were put in the receiver, and the back-titration was 3.4 c.c. of 0.01 N NaOH.

The acid neutralised by the ammonia was therefore  $10 - 3.4 - 0.2$  or  $6.4$  c c of  $0.01$  N

The nitrogen from  $\frac{5}{31}$  c c of serum was therefore  $6.4 \times 0.14$ , or  $0.896$  mgm

$100$  c c of serum therefore contained  $0.896 \times \frac{31}{5} \times 100$  mgm, or  $0.556$  gm of N. But the N P.N. accounted for  $0.060$  gm of this. The albumin N therefore amounted to  $0.556 - 0.060$ , or  $0.496$  gm

The albumin was therefore  $0.496 \times 6.25$ , or  $3.10$  per cent

The Globulins, by difference, therefore equalled  $5.93 - 3.10$ , or  $2.83$  per cent

Albumin	3.10 per cent
Globulins	2.83   ,,
Total proteins	5.93   ,,

#### (8) Example of Calculations for Plasma

**For Fibrinogen** The blank on  $1$  c c of water was  $0.15$  c c of  $0.01$  N ( $10 - 9.85$  c c). The fibrin was obtained from  $1$  c c of plasma (p 371),  $10$  c c of  $0.01$  N  $H_2SO_4$  were put in the receiver, and the back titration was  $6.2$  c c of  $0.01$  N NaOH.

The acid neutralised by the ammonia was therefore  $10 - 6.2 - 0.15 = 3.65$  c c of  $0.01$  N.

$1$  c c of plasma therefore yielded  $3.65 \times 0.14$  or  $0.511$  mgm of fibrin N.

$100$  c c of plasma therefore contained  $51.1 \times 6.25$  mgm, or  $0.32$  gm of fibrin.

**For Albumin + Globulin** (Albumin N + Globulin N + N P.N.) The blood urea was  $47$ , from which the N P.N. was calculated as  $42$  mgm per  $100$  c c (see p 373). The blank on  $3$  c c of a mixture of  $28$  c c of physiological saline,  $1$  c c of water and  $1$  c c of calcium chloride solution was  $10 - 9.8$ , or  $0.2$  c c of  $0.01$  N. The  $3$  c c of fibrin filtrate is equivalent to  $0.1$  c c of plasma, and  $10$  c c of  $0.01$  N acid were used, the back titration being  $2.1$  c c of  $0.01$  N NaOH.

The acid neutralised by the ammonia was therefore  $10 - 2.1 - 0.2 = 7.7$  c c of  $0.01$  N.

The nitrogen from  $0.1$  c c of plasma was therefore  $7.7 \times 0.14$ , or  $1.078$  mgm.

$100$  c c of plasma therefore contained  $1.078 \times 1,000$  mgm, or  $1.078$  gm of N, from which must be deducted the  $0.042$  gm of N P.N.

The albumin N + globulin N was therefore  $1.078 - 0.042$  or  $1.036$  gm per cent.

The albumin + globulin was therefore  $1.036 \times 6.25$ , or  $6.48$  per cent.

**For Albumin** (Albumin N + N P.N.) The calculated N P.N.

was 42 mgm, as above. The blank on 5 c.c. of 22 per cent  $\text{Na}_2\text{SO}_4$  was 10 — 9.8, or 0.2 c.c. of 0.01 N. The equivalent of  $\frac{5}{31}$  c.c. of plasma (see p. 372) was employed, 10 c.c. of 0.01 N  $\text{H}_2\text{SO}_4$  were put in the receiver, and the back titration was 2.0 c.c. of 0.01 N NaOH.

The acid neutralised by the ammonia was therefore 10 — 2.0 — 0.2, or 7.8 c.c. of 0.01 N.

The nitrogen from  $\frac{5}{31}$  c.c. of plasma was therefore  $7.8 \times 0.14$ , or 1.092 mgm.

100 c.c. of plasma therefore contained  $1.092 \times \frac{31}{5} \times 100$  mgm, or 0.677 gm. of N, from which has to be subtracted the 0.042 per cent of N.P.N. The albumin N therefore amounted to 0.677 — 0.042, or 0.635 gm., and hence the albumin was  $0.635 \times 6.25$ , or 3.97 per cent.

The Globulins, by difference, therefore equalled 6.48 — 3.97, or 2.51 per cent.

Fibrinogen	0.32 per cent
Albumin	3.97 „
Globulins	2.51 „
Total proteins	6.80 „

### The Biuret Method

The following description is for use with the permanent glass standards and Lovibond comparator supplied by Messrs The Tintometer Ltd. or Messrs The British Drug Houses Ltd. (for illustration of comparator, see p. 296). This particular technique is selected because it is so useful in routine clinical work, but the description applies equally well if *Fine's diluted serum* containing 0.24 per cent protein, or *Hiller's pure biuret solution* be used as the standard, comparison between standard and unknown being made in a colorimeter. Naturally the colorimeter gives a little more precision than the comparator, and the appropriate calculations have to be introduced. The comparator technique with the same coloured glasses may be used also for proteins in urine (p. 35), in cerebrospinal fluid (p. 406), in milk (p. 419) and in ascitic fluids (p. 411). The glasses are in two discs, labelled 20 to 180 and 200 to 360 mgm. respectively (steps of 20 mgm.), which give direct readings for total protein of urine and cerebrospinal fluid, but which have to be multiplied by factors (see below) in serum or plasma estimations.

**Principle.** As for urinary proteins (see p. 36). In the differential estimation in serum, globulins are precipitated by half saturation with ammonium sulphate, and the albumin is estimated in the filtrate by the biuret technique. In plasma fibrinogen is precipitated with the globulins, so that again the estimation on the filtrate gives albumin, fibrin(ogen) is determined separately after recalcification.



of plasma In both serum and plasma the globulins are calculated by difference Howe's method of fractionation with 22 per cent sodium sulphate (*cf* p 369) may be used instead of ammonium sulphate but it is more tedious and sometimes gives different results (*cf* Fine and footnote p 369)

The glass standards were prepared as described for urinary proteins (see p 36)

**Reagents and Centrifuge Tubes** The tubes, the trichloroacetic acid sodium hydroxide copper sulphate and saturated ammonium sulphate solutions are described under urinary proteins (see p 37)

27.79 per cent ammonium sulphate solution (A R)

0.85 per cent sodium chloride solution

Calcium chloride solution, 2.5 gm of anhydrous  $\text{CaCl}_2$  or 5 gm of crystalline  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$  in water to 100 c c

**Technique for Total Protein in Serum (Albumin + Globulin), or in Plasma (Albumin + Globulin + Fibrinogen)** Mix in a graduated centrifuge tube by inverting repeatedly, but without vigorous shaking lest some precipitate become entangled in froth, and be not thrown down when centrifuged

Serum or plasma	0.2 c c
Water	4.8 "
10 per cent trichloroacetic acid	5.0 "

Allow to stand for a few minutes until the precipitate clumps Centrifuge thoroughly and decant the supernatant fluid as completely as possible by inverting the tube carefully and wiping the mouth with filter paper

Add to the precipitate 1 or 2 c c of water and 1 c c of 30 per cent NaOH, shake till the protein has dissolved

Add 1 c c of 5 per cent crystalline copper sulphate solution, and water to exactly 10 c c Mix thoroughly for at least one minute, and centrifuge well until all the precipitate of cuprous hydroxide has been thrown down

Transfer the clear supernatant fluid to a comparator tube, which place in the right hand recess of the comparator Match against the glasses in the disc (200 to 360 mgm disc as a rule)

Total protein = reading  $\times$  25 mgm per 100 c c

$$= \text{reading} \times \frac{25}{1,000} = \frac{\text{reading}}{40} \text{ gm per 100 c c}$$

**Technique for Separate Determination of Albumin in Serum or Plasma** Mix in a test tube

Serum or plasma	0.5 c c
27.79 per cent ammonium sulphate	9.5 "

Filter through two thicknesses of No. 44 Whatman filter paper, and, if necessary, refilter till clear

Place 5 c c of the filtrate ( $\equiv$  0.25 c c of serum or plasma) in a graduated centrifuge tube, continue exactly as for total protein and read in the comparator

$$\begin{aligned}\text{Albumin} &= \text{reading} \times 20 \text{ mgm per } 100 \text{ c c} \\ &= \text{reading} \times \frac{20}{1,000} = \frac{\text{reading}}{50} \text{ gm per } 100 \text{ c c}\end{aligned}$$

**Technique for Fibrinogen of Plasma** Mix in a boiling tube or small beaker

0.85 per cent NaCl	28 c c
Plasma	1 "
Calcium chloride solution (see Reagents)	1 "

and continue as described on p 371 in order to separate and press the clot in a folded filter paper. Transfer the clot to a graduated centrifuge tube

Add 1 or 2 c c of water and 1 c c of 30 per cent NaOH, and place in a boiling water bath until solution is complete. Cool. Add 1 c c of 5 per cent  $\text{CuSO}_4$  solution and water to exactly 10 c c. Mix for one minute, centrifuge, transfer the supernatant fluid to the comparator, and read

$$\begin{aligned}\text{Fibrin(ogen)} &= \text{reading} \times 5 \text{ mgm per } 100 \text{ c c} \\ &= \text{reading} \times \frac{5}{1,000} = \frac{\text{reading}}{200} \text{ gm per } 100 \text{ c c}\end{aligned}$$

**Calculation of Globulin** For serum globulin = total protein — albumin. For plasma globulin = total protein — albumin — fibrin(ogen)

**Remarks** It is important to avoid contamination by ammonia of the laboratory atmosphere, and to remove as completely as possible the supernatant fluid in the differential estimations, as explained under urinary proteins (see p 38)

### References

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## SODIUM

The sodium of the blood is in the plasma, there being little or none within the red cells. Potassium, on the other hand, is mainly intracellular in both blood and tissues. For normal values see p 336.

Sodium determinations have become of clinical interest and importance since Loeb's demonstration that the blood-sodium is often lowered in Addison's disease. It has been suggested that the suprarenals are important in controlling the sodium, just as the parathyroids are in controlling the calcium of the blood. This new view throws stress on the sodium itself, whereas previously attention was focussed on the chloride and bicarbonate, and their relative proportions, and the sodium with which they are almost exclusively combined was seldom considered. In clinical work serum is nearly always used for the estimation.

The serum sodium is low, down to 250 mgm per 100 c.c. and occasionally less, in the acute crises of Addison's disease, but in the chronic phases it is often only slightly depressed and may be within the range of health. The test is therefore of only limited value as an aid to diagnosis. In Addison's disease the low sodium is accompanied by a raised potassium content of the serum and generally also by a rise in the blood urea (*cf* Allott).

The lowering of the serum sodium is caused by excessive excretion of sodium salts in the urine, due to the deficiency of the internal secretion of the adrenal cortex. Suitable doses of cortical extract will correct the deficiency, but the extract at present is expensive, so treatment by sodium chloride and other salts of sodium has been extensively employed with the idea of compensating to some degree the increased excretion by an increased intake of salt. Patients have been improved considerably by sodium chloride, but few can tolerate more than 10 gm daily (*cf* Graham), and this treatment has allowed the cortical extract to be reduced or stopped altogether for some time. Estimations of serum sodium are useful *in following the effects of treatment*.

Though in clinical work Addison's is the disease in which estimations of the serum sodium are most often requested, a fall in the sodium may occur in many other conditions, *e.g.*, intestinal obstruction, acute gastro enteritis, diabetic coma, severe sweating if water intake is unrestricted (miners' cramp), etc. The concentration of sodium in the blood is associated intimately with that of chloride, bicarbonate and water, and in disease not only is team work required to undertake all the necessary analyses, but when the analyses are completed it is often difficult to disentangle the possible effects of complicating factors, such as acidosis, alkalosis, "partial starvation," anæmia, hyperglycæmia, and so on. McCance has given a masterly review of this complicated subject, and has

studied experimental salt deficiency in normal man under controlled conditions

### Colorimetric Determination of Serum Sodium

(Modification of method of McCance and Shipp, omitting removal of phosphate, which is insignificant in serum)

**Principle.** The proteins are precipitated by trichloroacetic acid. The sodium in an aliquot part of the supernatant fluid is precipitated as sodium zinc uranyl acetate ("triple acetate"). The precipitate is washed and treated with potassium ferrocyanide solution. The resulting plum red colour (due to uranyl ferrocyanide) is compared in a colorimeter with a standard NaCl solution which has been treated similarly and simultaneously.

**Special Reagents** *Alcoholic zinc uranyl acetate solution saturated with "triple acetate"* Dissolve 50 gm of uranyl acetate ( $\text{UO}_2(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$ ) in 250 c c of boiling 4 per cent v/v acetic acid. Dissolve 150 gm of zinc acetate,  $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$  in 250 c c of boiling 2 per cent v/v acetic acid. Mix the two solutions whilst boiling. Bring the mixture just to the boil again. Stand overnight and filter if necessary. To the filtrate add an equal volume of absolute alcohol. Stand for forty eight hours at  $0^\circ \text{C}$ , filter, and then store at  $0^\circ \text{C}$  in a glass stoppered bottle until required, and filter or centrifuge enough for the test(s) just before use to remove suspended sodium zinc uranyl acetate.

There is usually sufficient sodium salt as impurity in the solution of acetates to yield more than enough "triple acetate" to saturate the reagent. If not, triple acetate must be added to saturate.

*Ninety five per cent alcohol saturated with "triple acetate"* Add about 50 c c of the above alcoholic zinc uranyl acetate solution to about 100 c c of saturated NaCl in 50 per cent alcohol. Allow to stand. Decant most of the supernatant fluid. Centrifuge and wash the yellow precipitate of triple acetate thoroughly with 95 per cent alcohol. Suspend the washed precipitate in 95 per cent alcohol, transfer to a glass stoppered bottle with more of the alcohol to a total of about 500 c c. Shake thoroughly and store at  $0^\circ \text{C}$ . There must always be a precipitate of excess triple acetate at the bottom of the bottle. Centrifuge or filter enough for the test(s) just before use.

*Stock NaCl, 0.89 per cent* ( $\equiv 0.35$  per cent Na) made from pure dry analytical reagent.

*Standard NaCl, 0.178 per cent* ( $\equiv 0.07$  per cent Na) prepared from the stock NaCl by diluting 5 c c with water to 25 c c. Therefore 1 c c of standard solution  $\equiv 0.7$  mgm of Na.

**Technique** Mix in a centrifuge tube

{	Serum	1 c c
	Water	3 "
	20 per cent trichloroacetic acid A R	1 "

Centrifuge or filter

In another centrifuge tube mix

Supernatant fluid or filtrate	. 1 c c
(= 0.2 c c of serum)	
Alcoholic zinc uranyl solution	. 10 „

Stir with a sealed capillary pipette until the yellow precipitate of sodium zinc uranyl acetate forms. Stand at 0° C for one hour. Centrifuge well and drain off the supernatant fluid completely by inverting and wiping the mouth of the tube with filter paper. Shake the precipitate with 5 c c of 95 per cent alcohol saturated with triple acetate. Centrifuge and drain completely.

Add about 3 c c of distilled water and shake until solution is complete. Transfer quantitatively with more water to a 250 c c volumetric flask. Add 1 drop of glacial acetic acid, 0.5 c c of 20 per cent potassium ferrocyanide solution and water to the 250 c c mark. Mix and allow to stand for three minutes.

Compare in the colorimeter with 1 c c of standard NaCl solution (= 0.7 mgm Na) which has been treated in parallel with 10 c c of alcohol zinc uranyl solution and subsequently in the same way as the unknown.

**Calculation** Let S and U mm be the depth of the standard and unknown respectively. The standard contains 0.7 mgm Na. The unknown was prepared from the equivalent of 0.2 c c of serum.

Therefore Na in 0.2 c c of serum =  $\frac{S}{U} \times 0.7$  mgm

and 100 c c of serum contain  $\frac{S}{U} \times 0.7 \times \frac{100}{0.2}$  mgm

i.e., serum sodium =  $\frac{S}{U} \times 350$  mgm per 100 c c

**Blank** Take 0.5 c c of the standard NaCl solution (= 0.35 mgm Na) and 1.0 c c (= 0.7 mgm Na) and treat both as described above. Set the more concentrated at 20 mm and match the more dilute against it, the reading of the latter should be 40 mm, if it is less than 40, one or more of the reagents contains Na as impurity.

### Gravimetric Determination of Serum Sodium

(Method of Butler and Tufhull, but substituting the alcoholic solution of zinc uranyl acetate described above in the colorimetric method.)

**Principle** The serum is digested with sulphuric and nitric acids to destroy organic matter. The sodium in the digest is precipitated as sodium zinc uranyl acetate ("triple acetate"). The precipitate is filtered off in a Jena filter crucible, washed, dried and weighed.

**Special Reagents** These are the same as described for the colorimetric method (p. 379), viz., *alcoholic zinc uranyl solution saturated with triple acetate*, and *95 per cent alcohol saturated with triple acetate*, but are kept at room temperature.

**Technique.** In a Pyrex tube (200 × 25 mm) mix

Serum	1 c c
4 N $H_2SO_4$ <sup>1</sup>	1 "
Concentrated $HNO_3$	0.5 "
A small quartz crystal, or a Pyrex glass bead	

Digest until charring appears, when remove the flame and add carefully down the side of the tube 3 or 4 drops of concentrated  $HNO_3$  or of superoxol. Digest again. If the solution does not clear, repeat the addition of  $HNO_3$ , or of  $H_2O_2$ , and the heating. When the solution has cleared, heat for a further five to ten minutes.

Cool and add 4 or 5 drops of water. Pour into about 15 c c of alcoholic zinc uranyl acetate solution in a weighed Jena glass filter crucible (Fig. 68).

A suitable Jena glass filter crucible is size No. 2 with a capacity of 30 c c and a porosity 1 G 4. It is cleaned and dried with alcohol and ether, applying suction, it is put in a desiccator for half an hour and then weighed.

A solid rubber bung is inserted into the crucible from below, this prevents any solution passing through the filter and serves as a footpiece (Fig. 68, b).

Rinse the pyrex tube with three portions each of 0.5 c c of water, and then with two portions each of 3 c c of the alcoholic zinc uranyl solution transferring to the filter crucible.

Stir the contents of the crucible with a sealed capillary pipette until the yellow precipitate of triple acetate appears, and continue stirring for a further period of about five minutes.

Wash the stirrer with 3 c c of the alcoholic zinc uranyl solution as it is withdrawn. Cover the filter with a watch glass (Fig. 68, b) and set aside for one hour at room temperature.

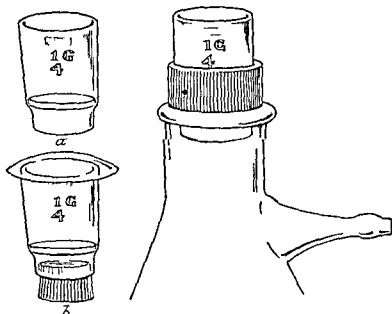


FIG. 68 Jena glass filter crucible. a Plain crucible. b, Rubber bung inserted from below. c Fitted in rubber collar in suction flask.

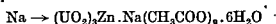
<sup>1</sup> An approximately 4N solution may be prepared by diluting 28 c c of concentrated  $H_2SO_4$  with water to 250 c c.

Remove the rubber bung, place the filter in a rubber collar in a suction flask and apply suction (Fig. 68, c). Wash the precipitate with five portions each of 2 c.c. of 95 per cent. alcohol saturated with triple acetate, being careful to wash the sides of the filter.

Wash with two portions each of 5 c.c. of ether, suck dry, desiccate for half an hour and weigh.

Blank. Perform a blank test in parallel, using 1 c.c. of water instead of serum.

Calculation.



23 mgm. of Na yield 1,537 mgm. of triple acetate precipitate.

Therefore 1 mgm. of precipitate is derived from  $\frac{23}{1,537}$  mgm. of Na.

Hence 1 c.c. of serum contains sodium to the extent of

$$\frac{23}{1,537} (\text{serum precipitate} - \text{blank precipitate})$$

and 100 c.c. of serum contain  $\frac{2,300}{1,537}$  (serum precipitate — blank precipitate) mgm. of Na.

Wherefore the serum sodium equals 1.495 (serum precipitate — blank precipitate) mgm. per 100 c.c.

Example of Calculation. Precipitate from 1 c.c. of serum = 225 mgm., and from 1 c.c. of water (blank test) = 5 mgm. The serum sodium was therefore 1.495 (225 — 5), or 329 mgm. per 100 c.c.

Note Weinbach (*J. Biol. Chem.*, 1935, 110, 95) has described a micro-modification of the above method (0.1 c.c. of serum), in which the triple acetate precipitate is dissolved in water and titrated with 0.02N. sodium hydroxide and phenolphthalein instead of being weighed.

### References

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## SUGAR (see Chapter VII)

## UREA

Methods of estimating blood urea have been given in Chapter V, where also the findings in renal and many other diseases, and in health, have been discussed. The conditions in which the urea content is raised or lowered, however, may conveniently be summarised

*Blood Urea Low*

Pregnancy  
Nephrosis (some cases)

*Blood Urea High*

Chronic nephritis (later stages)  
Renal infantilism  
Bilateral congenital cystic kidneys  
Mercury (bichloride) poisoning and lead poisoning (some cases)  
Acute nephritis (some cases)  
Gross obstruction in urinary tract (Enlarged prostate      Surgical kidney due to stone, etc)  
Gross destruction of kidneys (Tuberculosis      Stone      New growth, etc)  
Severe bilateral pyelitis, pyelonephritis, hydronephrosis, pyonephrosis, etc  
Severe anhydræmia (Infective diarrhoea of infants      Cholera      Extensive burns      Pulmonary oedema, *e.g.*, war gas poisoning, etc)  
Failing circulation (Severe cardiac disease      Terminal stage of many diseases)  
Due to gross loss of chlorides (Severe vomiting and/or diarrhoea      Intestinal obstruction      Pyloric obstruction      Diabetic coma (some cases)      Severe burns      Infantile diarrhoea      Cholera, etc)

In severe hæmatemesis and melæna

The importance of estimating the concentration of urea in the urine, as well as in the blood, in attempting to differentiate those cases in which a raised blood urea is not due to a renal lesion, has already been discussed in Chapter V. The increase in the blood urea is readily explained when there is gross renal damage or a



gross obstruction of the urinary tract, but in some diseases more than one factor may be responsible for the high blood urea, and it is not always possible to assess the relative importance of the different factors, or even to decide to what extent renal or non renal influences are responsible. Thus in infantile diarrhoea a raised blood urea might be due to the anhydræmia resulting from a loss of fluids by vomiting and diarrhoea, or due to a loss of chlorides from the same causes, with a resulting disturbance of the distribution of electrolytes and a compensatory retention of non protein nitrogenous bodies, or due (in part) to damage of the kidneys by the action of circulating toxins. If the urine contains no protein and nothing abnormal in the centrifuged deposit, the kidneys can be exonerated, but if the urine is not natural it may be difficult or impossible to assess the importance and extent of renal damage. Stress has been laid at different times and in varying degree on each of the three possible factors. The same or similar difficulties are encountered in certain cases of intestinal obstruction, general peritonitis, diabetes mellitus, lobar pneumonia, extensive burns, etc.

In pregnancy—particularly in the later months—the blood urea falls to a level a little lower than that in existence before and after this state so that a value exceeding 40 mgm per 100 c c during pregnancy is definitely pathological.

A rise in the blood urea of patients with hæmatemesis and melæna has been recorded by several observers and it is generally agreed that the higher the blood urea the more severe is the hæmorrhage. Witts (*Brit Med J* 1937, 1, 847) concludes that with a blood urea above 75 mgm per 100 c c the hæmorrhage is likely to have been severe, and finds the estimation particularly useful in the first few days. The cause of the rise is not known definitely, but Witts suggests that it is due to a depression of renal function, owing to the fall in blood volume and in blood flow through the kidneys.

## URIC ACID

Historically it is interesting that uric acid was the first blood analysis ever attempted. Sir A. B. Garrod in 1848 published the figures of the quantities of uric acid or of urates he obtained from the relatively large volumes of blood which were available, bleeding still being widely practised in his days. It is remarkable how close were his results to those obtained by modern methods.

The normal values are given on p. 336. The findings in renal disease have been discussed in Chapter V, where also was mentioned the slight rise which occurs during the first three or four days of life. In leukaemia the blood uric acid rises, presumably due to increased formation, associated with the increase of white blood corpuscles. Values of the order of 10 mgm per 100 c.c. have been reported in this disease.

The destruction of the nuclei of normoblasts seems to be the chief source of endogenous uric acid in health. The blood uric acid rises for two or three days during the successful treatment of pernicious anaemia with liver, this occurs before the reticulocyte response, and the extra uric acid is not derived from the liver extract (see Witts for references). Lennox has shown that the blood uric acid is raised by fasting for forty-eight hours or more, but this is seldom of clinical importance.

Estimations of uric acid are most frequently made in gout or suspected gout, and are often very useful. Several points, however, must be remembered in interpreting the results. In gout the values rarely, if ever, exceed 10 mgm per 100 c.c., whereas in advanced chronic interstitial nephritis figures as high as 20 mgm have been recorded. Moreover, in lesser grades of renal damage of many kinds, values of the same order as commonly encountered in gout (5 to 9 mgm) are frequently met with, when there is nitrogen retention. Chronic nephritis may coexist with gout or with other arthritic diseases, and more especially in the elderly subjects in whom the diagnosis between gout and other articular lesions commonly arises. A blood uric acid within normal limits does not exclude the diagnosis of gout. There is often a slight rise (of the order of 1 mgm) during the gouty attacks, but, as a rule, the increase is not striking, so that little help is obtained by waiting for an attack before taking blood for analysis. In non-gouty arthritis, unaccompanied by nephritis, the blood uric acid is usually normal, though, judging from the literature, it is unwise to conclude that hyperuricaemia (in the absence of kidney disease) necessarily proves that the arthritis is due to gout.

To summarise, hyperuricaemia occurs in the nitrogen retention of renal diseases, in gout (most cases), in non-gouty arthritis (few cases), and in leukaemia. To this list should be added eclampsia (some cases) and pneumonia (some cases).

Finally, the estimations are of value in following the effects of the treatment of gout by certain drugs (e.g., phenyl cinchoninic acid (atophan, phenoquin, or cinchophen), salicylates, aspirin, etc.) which cause an increase in the urinary output of uric acid and a lowering of the uric acid of the blood. Colchicum has no influence on the excretion of uric acid in the urine.

### Technical

Most modern methods are modifications of Folin and Denis's original method, and the majority are published in the *Journal of Biological Chemistry*, and are discussed critically in Peters and Van Slyke's second volume. The method described below is essentially that of Folin (see Folin and Trimble), the writer has not had sufficient experience of Folin's final technique (Folin, 1933) to feel justified at present in adopting it as a routine.

**Principle** The proteins of a known volume of blood are precipitated by sodium tungstate and sulphuric acid. An aliquot part of the protein free filtrate is treated with phosphotungstic acid and sodium cyanide under prescribed conditions, and the resulting blue colour, due to reduction of phosphotungstic acid by uric acid, is compared in a colorimeter with that of a standard solution of uric acid similarly treated.

✓ **Reagents** (1) *Uric Acid Reagent* In a 500 c.c. flask place 50 c.c. of 89 per cent w/w phosphoric acid, S.G. 1.75, and 160 c.c. of distilled water. Heat, and when boiling add 100 gm. of pure sodium tungstate  $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ . Boil gently for one hour, placing a funnel in the mouth of the flask to act as a condenser.

Into a 1,000 c.c. beaker transfer 25 gm. of lithium carbonate, 50 c.c. of 89 per cent phosphoric acid, and 200 c.c. of distilled water. Boil off the carbon dioxide and cool.

Mix the two solutions and dilute to 1,000 c.c.

(2) *Sodium Cyanide Solution* Fifteen per cent sodium cyanide in 0.1 N sodium hydroxide. This keeps for about a month. It is very poisonous and should be measured from a burette.

✓ (3) *Uric Acid Stock Solution* Transfer 100 mgm. of pure uric acid to a clean 100 c.c. beaker. Dissolve 50 mgm. of lithium carbonate in 20 c.c. of water in a boiling tube or small beaker, heat to 60° or 70° C., and pour this warm alkali on to the uric acid. Stir until the uric acid is completely dissolved. Add 5 c.c. of 40 per cent formaldehyde, and 1 c.c. of 50 per cent acetic acid. Mix and transfer quantitatively to a 100 c.c. volumetric flask. Make up to volume, mix and transfer to a glass stoppered bottle. One c.c. contains 1 mgm. of uric acid.

(4) *Uric Acid Standard* Transfer 1 c.c. of the uric acid stock solution to a 250 c.c. volumetric flask. Half fill the flask with water. Add 10 c.c. of 2/3 N sulphuric acid, and 1 c.c. (but no more) of 40 per cent formaldehyde. Dilute to the mark with water, mix and date. This solution should keep for at least one month. Five c.c. contain 0.02 mgm. of uric acid.

(5) *Forty per cent Urea* This is used to prevent turbidity in

the treated standard and unknown (*cf* Folin, *J Biol Chem*, 1930, 86, 179, and Rimington, *Biochem J*, 1930, 24, 1114)

**Technique.** Venous blood is obtained in the usual way, and the proteins of whole blood, plasma or serum are precipitated with sodium tungstate and sulphuric acid as previously described (see p 332). If oxalate or citrate is used as anticoagulant, an excess should be avoided. Taking whole blood as an example, mix

Blood	2 c c
Water	14 „
10 per cent sodium tungstate	2 „

and add 2 c c of 2/3 N sulphuric acid very slowly drop by drop with constant shaking, in order to avoid local zones of excessive acidity in which the uric acid might be precipitated, and adsorbed on to, or occluded in, the protein precipitate. Stand a few minutes until the precipitate clumps, and filter.

Two glass stoppered test tubes, or two Fohn's sugar tubes (p 143), each graduated at 12.5 c c, are required. In one (the "unknown") place 5 c c of the protein free filtrate. In the other (the "standard") place 5 c c of the uric acid standard. Add 2 c c of 40 per cent urea to each tube, then 2 c c of the cyanide solution, and finally 1 c c of the uric acid reagent. Mix the contents of each and allow to stand at room temperature for two minutes. Then place both tubes in a boiling water bath for 80 seconds. Cool and dilute to the 12.5 c c mark with water.

Compare the colours of unknown and standard in the colorimeter without delay, since fading may occur on standing.

**Calculation.** Let  $S$  be the reading of the standard, and  $U$  of the unknown. Let  $x$  be the mgm of uric acid in the 5 c c of protein free filtrate.

Since the 5 c c of the uric acid standard solution employed contain 0.02 mgm of uric acid,

$$x \times U = S \times 0.02,$$

$$\text{or} \quad x = \frac{S}{U} \times 0.02 \text{ mgm}$$

Since 5 c c of protein free filtrate correspond to 0.5 c c of blood,

$$0.5 \text{ c c of blood contains } \frac{S}{U} \times 0.02 \text{ mgm of uric acid}$$

$$1 \quad \text{,,} \quad \text{,,} \quad \text{,,} \quad \frac{S}{U} \times \frac{0.02}{0.5} \text{ mgm of uric acid}$$

$$100 \quad \text{,,} \quad \text{,,} \quad \text{contain } \frac{S}{U} \times \frac{0.02}{0.5} \times 100 \text{ mgm of uric acid,}$$

$$\text{or } 4 \times \frac{S}{U} \text{ mgm of uric acid}$$

The proportionality of colour is so good that readings covering a range of 2 to 8 mgm per 100 c c are dependable. Thus, if  $S$  is 20 mm, and  $U$  falls anywhere between 10 and 40 mm, there is no

need to prepare another standard the colour of which approximates to that of the unknown

**Blank** Transfer 5 c c of water, 2 c c of the cyanide solution, and 1 c c of the uric acid reagent to a test tube Mix and allow to stand at room temperature for two minutes The solution should remain colourless Heat in a boiling water bath for eighty seconds and cool Some colour is obtained To determine whether this colour does or does not materially affect the uric acid values in actual determinations perform tests with the uric acid standard solution as follows In one stoppered test tube graduated at 12.5 c c place 5 c c of the uric acid standard solution In another similar tube place 3 c c of the uric acid standard solution and 2 c c of water To each add 2 c c of 40 per cent urea 2 c c of the cyanide solution and 1 c c of the uric acid reagent Mix and stand for two minutes Heat in the boiling water bath for eighty seconds Cool and dilute to 12.5 c c Compare in the colorimeter If the cyanide is perfectly good the more dilute uric acid solution will give the theoretical reading of 33.3 mm when the more concentrated uric acid solution is set at 20 mm

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## VOLUME OF WHOLE-BLOOD AND PLASMA

There are two fairly trustworthy methods of determining the blood volumes of patients, the carbon monoxide method (Haldane and Smith) and the dye (vital red) method (*cf* Keith *et al*) The former gives results a little lower, the latter gives results a little higher than the actual blood volume, owing to transfusion of a portion of the dye into the lymph Lindeman has suggested a third method for cases of anæmia Using the dye method, healthy adults have about 50 c c of plasma and about 83 c c of whole blood per kilogramme of body-weight The normal range of variation, expressed in different ways, is recorded on p 336 The plasma volume is more constant than the whole blood volume (see table on p 390) In the obese the plasma volume per kilogramme of body weight is low For the values in infants and children, which reach the "adult level" at the age of four years, the reader is referred to a paper by Darrow *et al* Erlanger, and also Rowntree and Brown, give reviews of the subject, which is considered critically in excellent chapters in Peters and Van Slyke's two volumes

Estimations of the blood volume are important in studying hydræmia, an increase of the percentage of water in the blood, and anhydræmia, a decrease of the water per cent An increase of water per cent may be due to the addition of water or to the retention of water, *true hydræmic plethora*, or to a diminution of the percentage of solids in the blood, especially of the blood corpuscles or of the plasma proteins In *true hydræmic plethora* the blood volume is increased, in *hydræmia due to loss of solids* the blood volume may be unaltered or even diminished

Hæmoglobin determinations or estimations of the percentage of solids may be used as a rough measure of rapid changes in the blood volume over short periods, but such methods are not trustworthy over longer periods in which solids (*eg*, hæmoglobin (corpuscles) or plasma proteins) may have left the blood

The main diseases in which the whole blood volume is altered are listed below —

*Whole blood Volume Increased*

Polycythæmia

Nephritis with œdema (some cases)

*Whole blood Volume Decreased.*

Diabetic coma

Anæmias

Severe vomiting

Severe diarrhœa

Cholera

Infantile diarrhœa.

Severe shock

Severe burns

Pulmonary œdema due to war-gas poisoning

Lobar pneumonia (some cases)

Addison's disease

The table below, giving Bock's data for blood volume, is taken from Myers' book

*Data on Blood Volume. From Bock, partly Recalculated*

Condition.	Number of Cases	Total Plasma per cent of Body weight	Total Blood per cent of Body weight	Hemoglobin Calculated from G. Capacity per cent	Red Blood Cells in millions per c.mm.
Normal . .	5	5.1	8.2	16.4	4.8
Polycythæmia .	3	5.1	13.7	22.5	9.1
Pernicious anæmia .	7	4.9	5.7	5.8	1.6
Miscellaneous .	7	4.9	7.1	10.9	3.9
Diabetes .	8	4.8	7.3	16.3	4.6

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## CHAPTER XX

### CHEMICAL EXAMINATION OF THE CEREBROSPINAL FLUID

**Books** The reader will find an excellent account in Greenfield and Carmichael's *The Cerebrospinal Fluid in Clinical Diagnosis*, together with references to other works

Dana C L, and others, editors, *The Human Cerebrospinal Fluid* Vol IV of the publications of the Association for Research in Nervous and Mental Disease

Kafka's *Die Zerebrospinalflüssigkeit* gives an extensive bibliography

Katzenelbogen's *The Cerebrospinal Fluid and its Relation to the Blood*

THE cerebrospinal fluid is examined as an extension of the clinical investigation of the nervous system, and also in certain general diseases, *e g*, syphilis Diseases which alter the chemistry of the blood often also alter the chemistry of the cerebrospinal fluid, *e g*, diabetes, lobar pneumonia and kidney disease Certain blood constituents, however, do not pass or do not readily pass into the cerebrospinal fluid It follows, therefore, that changes in the blood may not be reflected by corresponding changes in the cerebrospinal fluid Thus in recent jaundice bilirubin is generally absent from the fluid, but when the hyperbilirubinæmia is of long standing the pigment may appear in the cerebrospinal fluid

A complete examination of the cerebrospinal fluid includes the manometric measurement of the pressure, observation of the general appearance, the cytology, bacteriology and chemistry of the fluid, together with certain special reactions such as the Wassermann test and Lange's colloidal gold test In this book description should be limited to the chemistry, but in the table on p 394 notes on cell counts have been included for reference In looking into the literature and the records of the hospitals in which he has worked, the author has been struck by the incompleteness of the quantitative data In well equipped institutions, protein, chlorides, sugar and urea could readily be estimated in the majority of cases as a routine, and such analyses would lead to more accurate knowledge It is essential to remember that qualitative tests (*e g*, for protein and sugar) can only reveal with certainty gross departures from the normal By purely qualitative means an experienced worker may be able to detect less gross variations, but he cannot readily convince his clinical colleagues unless he works quantitatively Incidentally, quantitative analysis would probably reveal such overlapping of the ranges of variation in different conditions, that not a few of the current ideas as to the value of these tests in particular diseases would have to be modified

#### WITHDRAWAL OF CEREBROSPINAL FLUID

The cerebrospinal fluid may be withdrawn by lumbar, cisternal or ventricular puncture The technique of these operations is well described in many books (*e g*, in that of Greenfield and Carmichael) The only points requiring mention here are those which may



influence chemical analysis. The greatest care should be taken to avoid contamination with blood. If the needle has been kept in spirit or carbolic acid it should be well rinsed in sterile physiological saline or sterile distilled water before use, because alcohol or phenol is apt to coagulate the proteins in the first portion of fluid passing through the needle. The lumbar puncture needle, with the stylet *in situ*, can hold but the merest trace of water or saline, so that no appreciable dilution error is introduced into the analysis. The possibility of such an error, however, is entirely eliminated by dry sterilising the needle. A convenient method is illustrated in Fig 69, and is exactly similar to that recommended for venepuncture needles (see Chapter XVII). It has the great advantage, too, that the needle is ready for immediate use, and can be carried ready sterilised to a distant house. After use it is thoroughly washed out with water, and then placed in concentrated carbolic acid or other suitable disinfectant for an hour or more to avoid any possible risk to the next patient. The disinfectant is washed out with distilled water, the needle is then rinsed with acetone to remove the water, placed in the tube, which is plugged with cotton wool, and sterilised, in the hot air oven. The acetone vaporises, leaving the needle quite dry.



FIG 69 Lumbar puncture needle sterilised in special tube

Fluids obtained simultaneously from the three sites ventricular, cisternal and lumbar, have slightly different compositions. Thus normal ventricular fluid is practically free from protein and cells, and has a lower specific gravity than lumbar fluid. The table shown below in illustration of the main differences in sugar content, is taken from a paper by Chevassut. Similar triple estimations of other ingredients would be valuable.

In the vast majority of patients fluid is obtained from the lumbar region, and subsequent description is restricted to lumbar fluid.

*Sugar (mgm per 100 c c)*

Blood	Ventricular Fluid	Cisternal Fluid.	Lumbar Fluid.
200	196	112	100
195	175	150	100
175	175	100	93
148	150	100	93
143	137	100	93
125	121	93	86
112	106	86	86
106	100	86	86
100	137	93	93

## THE NORMAL FLUID

The chief chemical characteristics of normal cerebrospinal fluid are tabulated below

	Normal *	Abnormal
Naked eye appearance .	Clear Bright Colourless  No coagulum	Turbid Dull Colourless or yellow (bilirubin) May be coagulum
Pressure	60 to 200 mm of C.s.f.	Above 200 mm of C.s.f.
Total protein	10 to 35 mgm per 100 c c	Above 35 mgm per 100 c c
Globulin test	Negative	May be positive
Sugar	45 to 100 mgm per 100 c c	{ Below 45 } mgm per 100 c c { Above 100 }
Qualitative test	Positive	{ Negative { Strongly positive
Chlorides (as NaCl)	700 to 760 mgm per 100 c c	{ Below 700 } mgm per 100 c c { Above 760 }
Urea	10 to 40 mgm per 100 c c	Above 40 mgm per 100 c c

Estimations of other constituents have been made, but the results either need extension and confirmation, or are of little value clinically. Much work (McCance and Watchorn, Herbert) has been done on the inorganic constituents and their concentrations relative to those in the blood serum, which is of great interest, the conclusion being reached that cerebrospinal fluid cannot be a simple filtrate from blood.

Ordinarily only the total protein is estimated. A differential estimation of albumin and globulins (and fibrin) is a relatively complicated procedure, and, as yet, has not been applied much in clinical investigations. Hewitt's findings for normal fluid are, albumin about 20 mgm and globulin about 3 mgm per 100 c c, with a ratio of albumin to globulin of about 8 to 1.

Both sugar and urea are of slightly lower concentration in the lumbar fluid than in the blood, whereas chlorides are distinctly higher in the fluid.

## PATHOLOGICAL VARIATIONS

The table on pp 394 to 398 summarises the results obtained in different diseases. This has been compiled in the main from the book by Greenfield and Carmichael, to which the reader is referred for fuller details (see also Carmichael, and Greenfield, 1928).

## Chemical Composition of Cerebrospinal Fluid in Disease

Disease	Quantitative mgm per 100 c c				Globulin 1 test	Cells per c mm	Type of Cell	Remarks
	Total Protein	Chlorides as NaCl	Sugar	Urea				
Normal . . .	10 to 35	700 to 760	45 to 100	10 to 40	Negative	0 to 3, even 5	Lymphocytes	Clear, colourless
meningitis, tuberculous .	30 to 400	700 to 500	Early stages normal, or +, later 15 to 45	10 to 40	Usually positive	20 to 1,000 + Usually 100 to 300	Lymph and polymorphs	Sometimes yellow.
" meningococcal .	100 to 400	750 to 600	0 to 45	10 to 40	Positive	1,000 to 2 000 +	Poly mostly	" "
" pneumococcal and streptococcal	100 to 200 +	750 to 600	0 to 45	10 to 40	Positive	Usually 1,000 + Occasionally few in very acute cases	Poly	" "
" staphylococcal .	100 to 200 +	750 to 600	0 to 45	10 to 40	Positive	Usually 1,000 + and purulent, occasionally Froin's syndrome	Poly	" "
" influenza! . . .	30 to 100 +	750 to 650	10 to 60	10 to 40	Positive	---	Poly mostly In some poly and lymph	" "
" typhoid . . .	100 to 200 +	750 to 600	0 to 45	10 to 40	Positive	Mild cases, 80 to 100 Severe, 1,000 +	Mild, lymph mostly Severe, poly mostly Lymph	" "
" mumps . . .	30 to 130	750 to 670	70 to 115	10 to 40	?	300 to 3 000	Usually poly	
" following disease of ear or nose	20 to 100 +	750 to 620	0 to 100	10 to 40	Positive or negative	15 to 5,000 +		

Irritation of meninges by serum therapy.	Increased	700 to 760	Present May be increased.	10 to 40	Usually negative.	Normal or little increased.	Lymph.
Influenza without meningitis .	10 to 40	Slightly reduced.	Diminished Not absent	10 to 40	Negative	100 to 400	Mostly lymph.
Typhoid without meningitis .	10 to 40	620 to 780	20 to 50	10 to 40	Negative	Normal or slight increase.	Lymph
Mumps without meningitis	10 to 40	700 to 760	45 to 100	10 to 40	Negative	Slight increase	Lymph.
Typhus . . . .	40 to 180	Slightly reduced or increased to 900	Normal or increased	40 to 250	—	7 to 270	Mostly lymph
Lateral sinus thrombosis . .	10 to 45	700 to 760 (dura not exposed)	45 to 100	10 to 40	Negative or positive.	4 to 50	Mostly lymph
Brain abscess . .	10 to 120	700 to 760 (dura not exposed by operation) 700 to 760	45 to 100, some above 100	10 to 40	Negative or positive	6 to 850	Poly. or lymph
" tumour . . . .	(Majority normal) 20 to 350		45 to 120 (Usually slightly increased)	10 to 40	Negative	(Majority normal) 0 to 80	Lymph Occasionally yellow.

## Chemical Composition of Cerebrospinal Fluid in Disease—continued

Diets.	Quantitative mgm per 100 c c				Globulin Test	Cells per c mm	Type of Cell	Remarks
	Total Protein.	Chlorides as NaCl	Sugar	Urea				
Cerebral hemorrhage or thrombosis	Commonly in excess	700 to 760	45 to 100 or slightly increased	10 to 40	Negative	Normal or sometimes increased	Lymph	Often blood and xantho chroma
Meningeal hemorrhage	10 to 100	700 to 760 (+ in renal inefficiency)	Slightly increased	10 to 40 (+ in renal inefficiency)	Negative	Increased (blood admixture)	Lymph and poly	Often blood and xantho chroma
Syphilis congenital (apart from G P and tabes)	10 to 35 (some over 35)	700 to 760	45 to 100	10 to 40	Negative Some positive	0 to 5 (some slightly increased)	Lymph	
" secondary neuro syphilis	25 to 50	—	—	10 to 40	Positive	10 to 100 (rarely 100 +)	Generally lymph	
" tertiary	50 to 150	700 to 760	18 to 62 Increased in some	10 to 40	Positive	50 to 500	Mostly lymph	
" G P I	30 to 60 40 to 100 Occasionally 100 +	700 to 760	25 to 62 Increased in some	10 to 40 10 to 60	Positive Strongly positive	10 to 30 50 to 500	Lymph Mostly lymph	
" tabes	20 to 60 Rarely to 100	700 to 760	45 to 100	10 to 60	Positive	10 to 80 Occasionally to 150	Mostly lymph	
Herpes zoster	10 to 60	700 to 760	45 to 100	10 to 40	Negative	0 to 500	Lymph	

Neuritis and neuralgia, inguinal	10 to 35 10 to 100	700 to 760 700 to 760	45 to 100 45 to 100	10 to 40 10 to 40	Negative Negative Rarely positive	0 to 5 0 to 8	Lymph Lymph	Sometimes yellow
" sciatica					Negative or negative	0 to 5	Lymph	Alcohol present in some.
" brachial neuritis	10 to 75 10 to 350	700 to 760 700 to 760 Increased if nephritis	45 to 100 45 to 110	10 to 40 10 to 40 Increased if nephritis 10 to 40	Negative Occa sionally positive Negative	0 to 5 Occasionally to 100	Lymph Lymph	Mercury may be found Trace lead may be found
" diphtheritic paralysis					0 to 5	0 to 5	Lymph	
" acute generalised poly- neuritis and Landry's paralysis	10 to 350 (Some show Fron's syndrome) 10 to 40	700 to 760	45 to 100	10 to 40	Usually negative	0 to 5	Lymph	
" alcoholic neuritis		700 to 760	45 to 100	10 to 40	Negative	0 to 5	Lymph	
" mercurial neuritis	10 to 35	700 to 760	45 to 100	10 to 40	Negative	0 to 5	Lymph	
" lead neuritis	10 to 35 Occasionally +	700 to 760	45 to 100	10 to 40	Negative Occa sionally positive Negative	0 to 5 Occasionally to 100	Lymph	
" arsenical neuritis	10 to 35	700 to 760	45 to 100	10 to 40	Negative or positive	0 to 5	Lymph	Often xantho- chromia
Spinal tumours	30 to 2,000 (Often Fron's syndrome)	700 to 760	45 to 100	10 to 40	Negative or positive	0 to 14	Lymph	
Diseminated sclerosis	Mostly 10 to 35 Few to 150	700 to 760	45 to 100	10 to 40	Negative or weak positive	Usually 0 to 10 Occasionally to 120	Lymph	
Syringomyelia Progressive muscular atrophy Subacute combined degeneration	10 to 60	700 to 760 (? subacute c d)	45 to 100	10 to 40	Negative	0 to 5	Lymph	

## Chemical Composition of Cerebrospinal Fluid in Disease—continued

Disease.	Quantitative    mgm per 100 c c				Globulin Test.	Cells per c.mm	Type of Cell	Remarks.
	Total Protein	Chlorides as NaCl	Sugar	Urea				
Polomyelitis, pre paralytic stage " first week " second and subsequent weeks.	10 to 30 30 to 60 20 to 300	700 to 760 700 to 760 700 to 760	45 to 100 45 to 100 45 to 100	10 to 40 10 to 40 10 to 40	Negative Negative Usually negative	30 to 2,000 10 to 1,500 Rapid fall	Most poly. Most lymph Lymph. and other mono nuclear.	
	Usually 10 to 40. Few to 100	700 to 760	50 to 150 Few number slightly raised	10 to 40	Negative Few weak positive	0 to 150	Lymph.	
	10 to 100	700 to 760	45 to 100	10 to 40	Negative or positive	0 to 2,000	Lymph.	
Typanosomiasis . . . .								
Arteriosclerosis and kidney diseases— (a) Normal kidney function (b) " Pure chloride retention " (c) " Nitrogenous retention "	10 to 120	700 to 760	45 to 100 (Few raised )	10 to 40	Negative.	0 to 5	Lymph.	
	10 to 120	780 to 820	45 to 100	10 to 40	Negative.	0 to 5	Lymph.	
	10 to 120	700 to 1,000	45 to 200 or more.	50 to 600	Negative.	0 to 5	Lymph.	
Diabetes (untreated). . . .	10 to 40	650 to 800	100 to 300 or more.	10 to 40 (Higher if anhy. dremia )	Negative.	0 to 5	Lymph.	Acetone often present.

Changes in pressure are of limited value. The pressure of the fluid depends on the intracranial venous pressure. An increase, therefore, is extremely common *e.g.*, in cerebral tumour, cerebral hæmorrhage, meningitis etc. A decrease is met with when the fluid in the lumbar theca is not in free communication with that in the cranium, *e.g.*, in spinal tumour, Pott's disease, etc. A gross increase in pressure is obviously present when the fluid really spurts out of the needle, but owing to variations in the size of needle, the risk of blocking the lumen, and so on, the only safe method is to measure the pressure with a manometer, the patient being horizontal.

Turbidity is usually due to increase in cells, or cells plus organisms. Rarely it is due almost entirely to bacteria. The cells may reach 200 or 300 per cmm. before the fluid is definitely opalescent. Sometimes the turbidity is due to small quantities of blood. The slight pinkish tinge may lead to its detection, and the presence of blood will be obvious from the colour of the sediment after centrifuging. If the presence of blood, and opalescence due to traces of alcohol or carbohic acid in the needle can be excluded, it may safely be said that turbidity is always pathological.

A fibrin coagulum is the most delicate index of the presence of fibrinogen. A fine fibrin web may not be noted, either because the examination is made too soon, or because the fluid is shaken up too much. A few cubic centimetres of the fluid should be put on one side, whilst the cells are counted, so as to see whether a fibrin web will form. After removal of the clot this fluid is used for chemical examination. A coagulum nearly always forms if the protein exceeds 100 mgm. It may form when the amount of total protein is less than 100 mgm. In suspected tuberculous meningitis the fibrin web should always be carefully examined for tubercle bacilli. In some cases no coagulum is formed, in spite of the presence of fibrinogen, owing to the absence of fibrin ferment. Clotting will occur if a drop of fresh serum is added and the fluid is incubated at 37° C. Sometimes a coagulum is due to accidental admixture with blood at lumbar puncture. If blood is absent, a coagulum is pathological.

**Colour.** The fluid may be pink or red, due to blood, or yellow, due to bilirubin. Blood may be obvious in the fresh fluid, or in the sediment after centrifuging. If the fluid above the red sediment in a centrifuged specimen is yellow, then some, at any rate, of the blood must have been in the fluid before puncture (except in long standing jaundice), because the yellow colour is due to bilirubin formed from blood shed into the fluid. Of course, if a large proportion of the fluid examined is blood derived from an accidental puncture of a vein, a yellow tinge may be due to gross admixture with plasma, but a small addition of fresh blood does not tinge the supernatant fluid. Usually when a vein is injured during puncture the fluid as it flows from a needle decreases steadily in redness, but this is not a certain method of differentiating "true" from "accidental" hæmorrhage. Sometimes when lumbar puncture is



repeated frequently (*e.g.*, daily), a series of coloured fluids may be obtained which, after centrifuging to remove cells, show varying colours from red to yellow, just as in a bruise, owing to the varying stages in the breakdown of oxyhæmoglobin to bilirubin. In other words, if a lumbar puncture has been made previously (within three weeks), little or no significance can be attached to the colour of the fluid.

That a yellow tint is due mostly, if not entirely, to bilirubin may be shown by performing Van den Bergh's indirect test. No other yellow substance which might be found in the cerebrospinal fluid would give this test. The writer has always found it positive in fluids which were obviously yellow, and judging by the number of Van den Bergh units and the depth of yellow, there is no need to postulate the existence of any pigment other than bilirubin.

A yellow tint is common in pathological fluids, *e.g.*, in meningitis of all kinds, in cerebral or meningeal hæmorrhage, in cerebral thrombosis, in cerebral or spinal tumours, and in polyneuritis. As already mentioned, in recent jaundice the fluid is rarely yellow, but may become tinged in long standing cases.

**Total Protein.** Increase of protein is the commonest abnormality of the cerebrospinal fluid. Protein should always be estimated quantitatively. The variations in different diseases are summarised on pp 394-398. Variations from 35 to 100 mgm per 100 c.c. are common, from 100 to 500 less common, and amounts over 500 mgm are rare. Increase of protein may or may not be accompanied by increase of cells (see table on pp 394-398).

Normal fluid contains albumin, pseudo globulin and a minute amount of euglobulin (*cf* p 393). Pathological fluids may contain larger quantities of euglobulin and fibrinogen, but the differential estimation of the proteins has not been applied extensively in clinical work as yet. In nearly all fluids albumin preponderates. By older analyses, in meningitis the ratio of albumin to globulin was of the order of 12 to 1, in general paralysis 7 to 3, whereas in spinal tumour the proportion might reach 2 to 1. Hewitt, using modern methods, gives the following ratios of albumin to globulin —

General paralysis (17 cases), between 0.9 : 1 and 2 : 1, av 1.3 : 1

Tabs (3 cases), between 2.7 : 1 and 3.4 : 1, av 3 : 1

Miscellaneous pathological (16 cases), between 3.0 : 1 and 12 : 1, av 6 : 1

Normally, about 8 : 1

The acetic anhydride and sulphuric acid test (see Greenfield and Carmichael 1927) is essentially an application of the Hopkins Adamkiewicz test for protein (Blix and Backlin, Duncan). The depth of colour, however, does not run strictly parallel with the total protein percentage, and it is possible that the ratio of albumin to globulin also influences the colour.

**Qualitative Test for Globulin.** This has been extensively employed in examinations of the cerebrospinal fluid, and, in the absence of contamination by blood, a positive reaction is always

pathological The results in different diseases are indicated on pp 394-398 Normal fluid contains traces of globulin (about 3 mgm per 100 c c, Hewitt), but not sufficient to give a positive qualitative test The test is very commonly positive in pathological fluids, in fact, almost always positive if the total protein exceeds 100 mgm Its chief value is perhaps in syphilitic lesions, in which it is generally positive, although the total protein is usually less than 100 mgm This can readily be understood from the fact that in syphilis the ratio of globulin to albumin is increased

**Glucose.** The reducing substance in cerebrospinal fluid is mostly, if not entirely, glucose The sugar content of the ventricular fluid is approximately the same as that of the blood, but the lumbar fluid sugar is almost always lower (table on p 392) The cause of this difference between ventricular and lumbar fluid sugar is not known It may be due to utilisation of sugar by the lining endothelial cells After a glucose meal the blood sugar rises much more rapidly than the cerebrospinal fluid sugar, but the cerebrospinal sugar does rise In other words, it takes time for the sugar from the blood to pass into the lumbar fluid Since the oscillations in blood sugar after a meal are generally rapid, it is easy to understand that variations in the lumbar fluid sugar are less marked and in fact have received little attention When hyperglycæmia is persistent, as in untreated diabetes mellitus, the cerebrospinal sugar also is raised, and very nearly, if not quite, to the same degree as the blood sugar Apart from diabetes the cerebrospinal sugar is increased in uræmia, and often when there is increased intracranial pressure, as in cerebral hæmorrhage cerebral tumour, and so on In short, the cerebrospinal-sugar follows approximately the blood sugar in these cases In meningitis there is the factor of increased intracranial pressure tending to raise the blood sugar, and, therefore, the cerebrospinal sugar, but there are other factors tending to lower the cerebrospinal sugar The figure obtained by analysis is, therefore, the algebraic sum of these influences In encephalitis it was at one time claimed that an increase in cerebrospinal sugar was an important diagnostic point, but recent work has modified this view In a fair number of patients with this disease there is a slight increase in sugar (up to 120 mgm), but a normal sugar by no means excludes the diagnosis

In acute meningitis the sugar is very often reduced or completely absent Further quantitative measurements are badly needed, but there are undoubtedly considerable variations in different stages of the disease

The chief factors responsible for a fall in the sugar content of the cerebrospinal fluid are (a) hypoglycæmia, (b) glycolysis by ferments formed from polymorphonuclear cells and (c) utilisation of the sugar as food by the organisms in the fluid Insulin hypoglycæmia, if prolonged, may cause a definite fall in the cerebrospinal sugar In meningitis (c) is often an undoubted factor, because it has been shown that lactic acid is formed, that the sugar may not be lowered if the invading organism does not ferment glucose, and that sugar

disappears rapidly from infected fluids, but only slowly from sterile fluids kept in the laboratory. The part played by cells is really not known, on the one hand it is possible to keep certain sterile fluids containing many cells without loss of sugar, on the other hand the turbid but sterile fluid in certain cases of brain abscess may contain little or no sugar.

Much interesting information would probably be derived from simultaneous blood and cerebrospinal sugar estimations. Thus in meningitis it has already been shown that hyperglycæmia may co exist with hypoglycorrhachia.

**Chlorides** Readings above normal (above 760 mgm) are most commonly encountered in renal inefficiency, and readings below normal (below 700 mgm) in meningitis. Chloride estimations have been particularly valuable in the diagnosis of tuberculous meningitis, in which disease very low values have been recorded, down to 500 mgm. They are also valuable in cerebral abscess and in other complications of infections of the ear and nose—so long as the dura mater has not been exposed by operation—in helping to decide whether there is generalised meningitis or not. A progressive lowering suggests that the subarachnoid space is becoming more widely involved. In acute lobar pneumonia the chlorides are lowered in the cerebrospinal fluid just as they are in the blood and the urine quite apart from the presence of meningitis. Probably the most potent factor in causing a fall in the cerebrospinal fluid chloride is a fall in blood chloride (*cf* Linder and Carmichael). Another possible factor in meningitis is the increase in meningeal permeability.

**Urea** Urea being a very diffusible substance, it is not surprising that the cerebrospinal fluid urea follows closely the blood urea, though the former is often slightly lower. Therefore, in routine work there is no point in estimating the cerebrospinal urea if the blood urea has already been determined and *vice versa*.

Urea estimations are chiefly requested in comatose or convulsed patients suspected of having uræmia, and lumbar puncture in these cases is often valuable not only in providing fluid for analysis, but also in relieving symptoms. The cerebrospinal urea is subject to the same limitations as the blood urea as an aid to diagnosis (see Chapter V). Thus severe anhydræmia, a failing circulation and so on all lead to raised values. It is in moribund patients particularly that difficulties arise and the results of urea estimations performed on cerebrospinal fluid removed after death must be interpreted with the greatest caution.

### From's Syndrome

When there is blockage of the subarachnoid space, the cerebrospinal fluid below the block approaches in composition to that of the blood plasma. The result is a great increase in protein, massive coagulation and often a yellow coloration, the cells varying from *nil* to many, depending on the cause of the block. The fluid is said to exhibit the syndrome of From. Greenfield has shown

that the essentials of From's syndrome are that the protein shall exceed 500 mgm, and that the fluid shall not be frankly purulent. A yellow colour is unessential. Absence of spontaneous coagulation is not uncommon, because although there is a large excess of fibrinogen, fibrin ferment may be absent, in such cases the addition of a drop of fresh serum causes instant coagulation. Sugar and chloride vary little from the normal.

The syndrome is found in chronic meningitis, especially syphilitic meningitis, tumours of the cord and its envelopes, spinal caries, epidural abscess (tuberculous or staphylococcal), and rarely in polyneuritis and Landry's paralysis. When the block is due to a tumour of the cord, it is possible that some of the protein in the loculated fluid comes from the tumour (*cf* McCance and Watchorn, 1934).

### Lange's Colloidal Reaction

Several colloidal tests have been devised—benzoin, Berlin blue, collargol, gamboge, gum mastic—but none of them is so sensitive as Lange's colloidal gold test.

Gold is precipitated from colloidal suspension by globulin, whereas albumin has a protective action. The ratio of albumin to globulin in cerebrospinal fluid appears to have an important influence on the precipitation of gold. Thus the maximum precipitation is obtained in general paralysis, in which the proportion of globulin is highest, but the reactions obtained in different diseases cannot be explained solely by differences in this ratio. Mellanby and Anwyl Davies suggest that it is euglobulin which causes precipitation, and pseudo globulin which keeps the gold in solution.

The test is performed by adding gold sol to serial dilutions of cerebrospinal fluid, and noting the change, if any, which occurs in each tube after twelve to twenty four hours.

- 0, denotes no change from the original bright cherry red
- 1, very slight change to deeper red, scarcely lilac
- 2, lilac to purple
- 3, deep blue
- 4, light blue with purplish precipitate
- 5, complete decolorisation of the supernatant fluid with heavy bluish precipitate

These changes simply represent increasing precipitation of the gold from the colloidal solution, and may be recorded as numerals or graphically, as shown below. The test itself is simple. It is the preparation of a suitable gold sol which presents the only difficulty (see under Technique). If the gold sol is a shade too acid, it will be too sensitive and may be precipitated even by normal cerebrospinal fluid, if it is a shade too alkaline it will not be sensitive enough and may not be precipitated even by a paretic fluid.

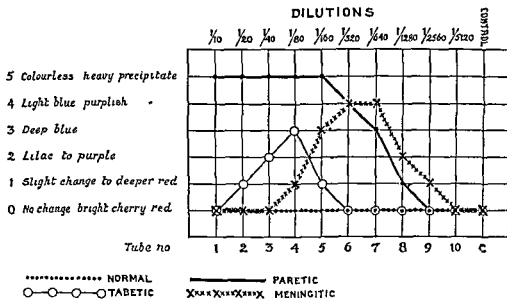


FIG 70 Langes colloidal gold test.

Typical curves obtained in health, in general paralysis, in tabes (or syphilitic meningitis), and acute meningitis respectively are illustrated (Fig 70) Reported as a series of numerals these would read —

Normal	000000000000
Paretic	55555431000
Tabetic (luetie)	01231000000
Meningitic	00013442100

Greenfield and Carmichael note the following (a) "In the presence of a negative Wassermann reaction in both blood and cerebrospinal fluid a 'paretic' type of gold sol curve is most often indicative of disseminated sclerosis, (b) that the persistence of a curve of the 'paretic' type during months of antisiphilic treatment is very strongly in favour of the diagnosis of 'general paralysis' "

## TECHNIQUE OF THE SIMPLER TESTS

### Protein

If the fluid is turbid it should be centrifuged to remove cells, and the supernatant fluid used for the determination

**Aufrecht's Picric Acid Method** This method has been described for urine in Chapter III, and is applied to cerebrospinal fluid in exactly the same way The objections to it are that it is only approximate, that it requires 4 c c of fluid, and that it is practically useless for fluids containing less than 100 mgm of protein per 100 c c It is serviceable for fluids containing much protein, but it is essential to follow the instructions with regard to the duration and rate of centrifuging (cf Chapter III) The method is not recommended

**Mestrezat's Diaphanometric Method** The protein is precipitated by trichloroacetic acid, and the resulting turbidity is compared with that of a series of standard protein solutions similarly treated

**The Test** Place 2 c.c. of cerebrospinal fluid in a test tube of the same bore and quality of glass as that of the standard tubes. Add 0.3 c.c. of 30 per cent trichloroacetic acid, mix, and place in a boiling water bath for about two minutes. Set aside for twenty minutes or longer, and then compare with the standards by viewing a window bar or Jaeger's test types through the turbid fluids

It is better to add the trichloroacetic acid *before* heating, since strings and large flakes of coagulated protein are less likely to form, but it is then essential to wait for twenty minutes or more for the mixture to cool, because the immediate effect of heating is often a reduction of the turbidity (due to the formation of acid metaprotein), which, however, increases on standing, so that finally it is the same as that obtained by first heating and then adding the acid. The method is an approximate one, because it is impossible to insure that all fluids and standards will yield the same state of protein aggregation, but it is roughly quantitative and a practical method for clinical work.

It is easy to distinguish between turbidities up to amounts corresponding to 50 or 60 mgm. of protein per 100 c.c. When the protein content is greater, it is best to dilute the fluid first with 1, 2, or more volumes of physiological saline, according to the rough estimate first made. Thus, if the first estimate is 200 mgm., another 1 c.c. of fluid is taken and 4 c.c. of saline are added. Two c.c. of the diluted fluid are treated with the trichloroacetic acid, the turbidity is matched and the resulting figure multiplied by 5.

**Preparation of Standard Tubes** Obtain 5 to 10 c.c. of fresh non-haemolysed normal human serum, and dilute it 1 in 10 with 0.85 per cent sodium chloride. Determine in duplicate or triplicate the total nitrogen by Kjeldahl's method in 5 c.c. portions, using 20 c.c. of N/10 sulphuric acid in the receiver to catch the ammonia. Calculate the protein content ( $N \times 6.25$ )<sup>1</sup> of the diluted serum, and dilute still further with physiological saline to exactly 0.1 per cent of protein (100 mgm. per 100 c.c.)

Fill one 5 c.c. microburette with the 0.1 per cent protein solution, and another with 0.85 per cent sodium chloride. Into a series of test tubes run in 2.0, 1.8, 1.6, 1.4, 1.2, 1.0, 0.8, 0.6, 0.5, 0.4, 0.3 and 0.2 c.c. of protein solution, and then 0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 c.c. of saline respectively. To each test tube add 0.3 c.c. of 30 per cent trichloroacetic acid, heat in a boiling water bath for about two minutes, and cool. Seal off each tube and mark with a diamond in sequence, 100, 90, 80, 70, 60, 50, 40, 30, 25, 20, 15 and 10 mgm. of protein per 100 c.c. Sterilise in the vaccine bath at 56° C. for one hour on three successive days.

<sup>1</sup> In normal serum the non protein nitrogen constitutes less than 4 per cent of the total nitrogen so that the total nitrogen may be regarded as the same as the protein nitrogen.

The tubes used for the standards and the tests must be of the same bore and type of glass "Cordite" tubes (tubes used for cordite testing) are very convenient ( $\frac{1}{2}$  in diameter) Each standard tube is first drawn out as shown in Fig 71 at (a), then filled (b), and then sealed (c) and (d) It is advisable to make a duplicate set to allow for breakages

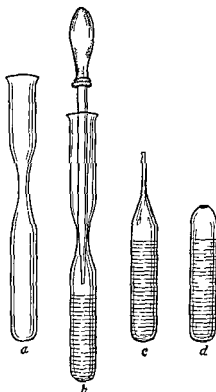


FIG 71 Preparation and sealing of a capsule containing fluid

**Biuret Method** This method is the same as that used for urine (Chapter III) but 2 c c of fluid are used with appropriate reductions of the volumes of reagents For convenience the steps are outlined briefly but reference should be made to Chapter III for full details

For *Total Proteins* mix in a graduated centrifuge tube —

Cerebrospinal fluid	2 c c
10 per cent trichloroacetic acid	2 „

Centrifuge and decant

Add to the precipitate

Water about	1 c c
30 per cent NaOH . . .	0.5 c c

and shake till the protein has dissolved Add 0.5 c c of 5 per cent crystalline copper sulphate solution, and water to exactly 4 c c Mix thoroughly for at least one minute, and centrifuge down completely the precipitate of cuprous hydroxide

Transfer the supernatant fluid to a Lovibond comparator (p 296), compare with the standard coloured glasses and read the answer directly

*Differential Estimation of Proteins* In pathological fluids a reasonably close estimate of the proportions of albumin and globulin may usually be made In brief, centrifuge or filter a mixture of

Cerebrospinal fluid	2 c c
52.8 per cent $(\text{NH}_4)_2\text{SO}_4$	2 „

In a graduated centrifuge tube mix

Supernatant fluid (or filtrate)	2 c c
10 per cent trichloroacetic acid	2 „

Centrifuge, decant completely and add to the precipitate the NaOH solution (0.5 c c), the  $\text{CuSO}_4$  solution (0.5 c c) with water to a total of 4 c c exactly as described above under 'Total Proteins' Read in the Lovibond comparator and multiply by 2 to obtain the albumin in mgm per 100 c c

Calculate the globulin by difference, total protein less albumin

*Other Methods* The protein in the cerebrospinal fluid may be precipitated by salicylsulphonic acid, and the resulting turbidity compared in a nephelometer, with the turbidity of a standard protein solution similarly treated (*cf* Denis and Ayer, also King and Haslewood) As in the trichloroacetic methods, the main objection is that the aggregates of protein molecules may not be the same in standard and unknown

The protein may be estimated accurately by the micro Kjeldahl method (*cf* Chapter XIX) The total nitrogen and total non protein nitrogen are each determined and the protein calculated as  $6.25 \times (\text{total nitrogen less non protein nitrogen})$  The method requires too much cerebrospinal fluid and too much time for routine clinical use It is recommended for research work

The total protein (Wu and Ling), and a differential estimation of the individual proteins (Hewitt), may be made by Wu's colorimetric method

### Globulin

Several qualitative tests for globulin have been described (see book of Greenfield and Carmichael), but for routine clinical work the Nonne Apelt reaction (phase I) meets most requirements To 1 volume of cerebrospinal fluid, 1 volume of a saturated solution of ammonium sulphate is added, e.g., 1 c c of each, and the mixture is shaken and set aside for three or more minutes Normal fluids remain clear or occasionally become very slightly opalescent Pathologically the mixture becomes slightly opalescent, opalescent, slightly turbid, or markedly turbid, or a precipitate occurs, thus giving a rough indication of the concentration of globulin present

It is a common procedure to perform this as a ring test, superimposing cerebrospinal fluid on saturated ammonium sulphate, and, though open to theoretical objections, in practice this gives substantially the same results as the Nonne Apelt reaction





silver nitrate and shake. Add 3 to 4 c c of pure acetone, mix and cool. Add 6 drops (0.3 c c) of saturated iron alum solution as indicator, and back titrate from a microburette with alcoholic ammonium thiocyanate solution of approximately half the strength of the silver nitrate and standardised against the latter. (A convenient solution is made by diluting ordinary aqueous N/20 thiocyanate with about 2 volumes of absolute alcohol.) The end point is reached when the mixture suddenly changes from a pale yellow to a pale brownish red.

*Example of Calculation* Two c c of alcoholic thiocyanate were equivalent to 1 c c of silver nitrate. Back titration was 0.56 c c of alcoholic thiocyanate. Therefore,  $\left(1 - \frac{0.56}{2}\right) = 0.72$  c c of silver nitrate was used to precipitate the chloride. From the equation on the previous page, 1 c c of 0.5814 per cent silver nitrate corresponds to 2 mgm of NaCl. Therefore, 0.72 c c of silver solution corresponds to 1.44 mgm of NaCl. Therefore, 0.2 c c of fluid contains 1.44 mgm of NaCl, and 100 c c of fluid contain 720 mgm.

### Sugar

**Qualitative Test** (a) Boil 1 c c of cerebrospinal fluid with 0.25 c c of Fehling's solution. Normal fluids give a heavy reddish-yellow precipitate, which on standing sinks to the bottom of the test tube, leaving the supernatant fluid pale blue.

(b) Boil a mixture of 1 c c of Benedict's qualitative reagent and 1 c c of fluid for two minutes, and allow to cool. Normal fluids give a small yellow precipitate and an opalescent greenish supernatant fluid.

**Quantitative Tests** Any of the blood sugar methods (see Chapter VII) may be used, with the following variations —

*Hagedorn and Jensen's Method* Use 0.2 c c of cerebrospinal fluid instead of 0.1 c c of blood. Calculate from the table in the usual way, and divide the resulting figure by 2.

✓ *Folin and Wu's Method* Take 1 c c of fluid, 8.5 c c of water, 0.25 c c of sodium tungstate solution and 0.25 c c of 2/3 N sulphuric acid. Mix, filter and proceed as for blood. The calculation is as for blood.

Alternatively mix 2 c c of fluid, 7 c c of water, 0.5 c c of tungstate and 0.5 c c of acid, filter and proceed as usual. The dilution is then 1 in 5 instead of 1 in 10, and calculation is made accordingly.

*Folin and Wu's Method Adapted for Smaller Quantities of Fluid* To 3.4 c c of water, add 0.2 c c of cerebrospinal fluid, 0.2 c c of sodium tungstate solution and 0.2 c c of 2/3 N sulphuric acid. Mix, filter and proceed as for blood. When making the calculation remember that 0.2 c c of fluid has been used, instead of 0.1 c c of blood.

### Urea

The methods used for estimating urea in blood (Chapter V) may be applied without modification to cerebrospinal fluid.

### Lange's Colloidal Gold Test

**Technique.** In a rack place eleven test-tubes. In the first tube put 1.8 c.c. of 0.4 per cent sodium chloride and 0.2 c.c. of cerebrospinal fluid. In each of tubes 2 to 11 place 1 c.c. of the saline. Mix the contents of No. 1 and transfer 1 c.c. to tube No. 2. Mix and transfer 1 c.c. to tube No. 3, and so on, finally discarding the 1 c.c. taken from No. 10. To No. 11, the control, add nothing. To each of tubes 1 to 11 add 5 c.c. of colloidal gold solution. Mix and set aside for twelve to twenty-four hours, at the end of which time the tubes are examined, and the results recorded as shown on p. 404.

**Preparation of Gold Solution** (Mellanby and Anwyl Davies). In a 250 c.c. beaker place 100 c.c. of *redistilled* water, and 1 c.c. of 1 per cent neutral potassium oxalate. Bring the mixture to the boil, add immediately 1 c.c. of 1 per cent gold chloride, and remove the flame. The fluid should be bright cherry red with practically no fluorescence. When 100 c.c. is viewed in bulk, it should be red without any blue tinge. If the colour is old rose, or if there is more than the slightest fluorescence, the fluid is useless.

Each new batch of gold sol should be tested against normal and known syphilitic cerebrospinal fluids to make sure that it is not too sensitive, and that it gives typical luetic and paretic curves. The stock normal and syphilitic cerebrospinal fluids may be stored in the ice chest, after heating at 55° C. to insure that they will remain sterile.

Either  $\text{AuCl}_3 \cdot \text{NaCl} \cdot 2\text{H}_2\text{O}$  or  $\text{AuCl}_3 \cdot \text{KCl} \cdot 2\text{H}_2\text{O}$  is recommended for preparing the 1 per cent gold chloride solution. The usual "gold chloride" obtained on the market is  $\text{AuCl}_3 \cdot 2\text{HCl}$ , or  $\text{AuCl}_3 \cdot \text{HCl} \cdot 3\text{H}_2\text{O}$ , and its solution is, of course, strongly acid, even after neutralisation with NaOH and litmus as external indicator, it is not satisfactory for preparation of the gold sol.

**Notes on the Test.** The water should be freshly redistilled from glass using no rubber connections. This is the essential part of the method. Since making it a routine invariably to redistil from glass, discarding the first 200 c.c. or so of distillate, all our difficulties have disappeared, the redistilled water may be kept in a paraffin-lined bottle with a bark cork for a week or so. The gold sols prepared with this redistilled water show very little variation in sensitivity.

Naturally all water used in the tests must be of the same good quality. Thus the sodium chloride, potassium oxalate, and gold chloride must each be made up in the redistilled water. The oxalate and gold chloride solutions will keep in glass stoppered bottles for several months. The saline will keep for several weeks if sterilised after withdrawing what is required for immediate use, but otherwise should be made up about once a week.

All glassware should be thoroughly cleaned by the technique described in the Appendix.

It is safest to use gold sol prepared freshly that same day, though it may keep for a few days in a glass stoppered bottle, when, however, it tends to become more sensitive.

There are on the market gold sols, prepared ready for use, but in the writer's experience these are too stable and are not recommended for Lange's test

### ASCITIC, CYSTIC, HYDROCELE, PLEURAL FLUIDS, etc

For the chemical composition of the fluids obtained from cysts (dermoid, hydatid, ovarian, etc), and of various effusions (ascites, chylous ascites, pseudochylous ascites, hydrocele, pleural fluid, etc), the reader is referred to Wells' *Chemical Pathology*. In routine clinical work requests for the chemical examination of these fluids are infrequent, for the results are seldom of practical clinical assistance, this is illustrated by data from Wells' book which are summarised below.

#### *Ascitic Fluids (after Wells)*

	Chylous Fluids	Chyliform Fluids	Pseudochylous Fluids
Cause of milkiness	Fat from chyle	Fat from degenerated cells	Lipoid globulin with or without fat
Total fat or ether soluble matter per cent	0.065 to 9.2 Av 1.65	0.1 to 4.3 Av 1.15	0.007 to 1.86 Av 0.25
Total protein per cent	0.9 to 7.7 Av 3.5	0.6 to 6.8 Av 3.0	0.1 to 4.2 Av 1.4

Clearly there is so much overlapping of results that the chemical findings are of little value. The cytology of the fluids taken in conjunction with the clinical findings usually suffices. It is possible that the administration by mouth of 100 mgm of Sudan III in 10 gm of butter (*cf* p 285) before tapping would assist in the recognition of a true chylous fluid.<sup>1</sup>

It is maintained that transudates usually have a specific gravity below 1015, and exudates above 1018 (the S.G. follows closely the protein percentages), but there are many exceptions to this statement. It is often difficult to decide whether a given fluid is an exudate or transudate, there being no definite line of demarcation either aetiological or chemically.

The technique of the analyses is generally the same as for blood, the biuret method (*cf* p 375) is useful for proteins.

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<sup>1</sup> Dr P. E. T. Hancock informs me (July, 1937) that he has tried this with success, the Sudan III appeared in the chylous ascitic fluid in about three hours.

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## CHAPTER XXI

### MILK ANALYSIS

**Books** Milk and its analysis is described in most books of physiological chemistry, *eg*, those of Hawk and Bergem and of Hammarsten and Hedin (trans Mandel)

Fuller information is contained in the following special manuals and articles —

Thorpe's *Dictionary of Applied Chemistry* Article on "Milk," by H Droop Richmond, 1922, Vol IV, 362

"On the Variations in the Composition of Normal Human Milk" Gardner, J A and Fox, F W, *Practitioner*, 1925, 114, 153

Allen's *Commercial Organic Analysis*, Vol IX

Davies' *The Chemistry of Milk*

ANALYSES of human milk are requested in certain cases in which the infant does not thrive on the breast, or appears to suffer from indigestion after the feed. It must be admitted at once that, in the great majority of instances, such analyses reveal nothing abnormal and give no assistance to the clinician. Analyses of cow's milk are required occasionally to test the quality of the milk supply, or to test for adulteration. The chemical pathologist sometimes undertakes this work, which, however, is more often performed by an analytical chemist.

Physicians must know the composition of normal human and of cow's milk in order efficiently to supervise infant feeding.

In this chapter will be given the composition of milk and notes on the technique of some of the simple methods of analysis.

### THE COMPOSITION OF HUMAN MILK

The method by which the mother's milk is obtained influences the analytical results. The milk becomes richer as the feeding of the infant progresses. There are three methods of securing the samples. The breast may be emptied completely with the aid of a breast pump, and the whole or a sample of the mixed milk sent for analysis. This is obviously the most satisfactory, but is not always possible in practice. Alternatively, the middle third may be obtained in the following way. The mother is instructed to time several feeds so as to find out how long the infant takes to empty the breast. The infant is then put to the breast for a third of this time. Then without delay about 1 oz. of milk is removed with a breast pump, whereafter the infant is allowed to finish his feed. Lastly, some workers first of all remove 1 oz. of milk, then put the baby to feed at the breast, and then remove another ounce with a breast pump. The 2 oz. are mixed and analysed.



*Hammett's Analyses of Human Milk on the Third to the Eleventh Day after Parturition (Eight Cases)*  
*Results in gm per 100 gm*

Day of Parturition.	MAXIMUM				MINIMUM				AVERAGE			
	Total N	Protein.*	Fat	Lactose	Total N	Protein.*	Fat	Lactose	Total N	Protein.*	Fat	Lactose
3	0.908	5.79	12.70	6.49	0.286	1.82	1.92	2.31	0.552	3.52	4.34	5.43
5	0.364	2.32	4.26	6.86	0.203	1.29	1.89	4.15	0.274	1.74	2.88	6.08
7	0.301	1.91	5.79	6.71	0.216	1.38	1.81	5.70	0.254	1.61	3.80	6.21
9	0.307	1.93	5.11	6.86	0.220	1.40	2.28	5.76	0.264	1.69	3.70	6.32
11	0.267	1.70	5.02	6.81	0.184	1.18	1.92	5.37	0.229	1.46	3.38	6.42
Average	0.428	2.73	6.53	6.74	0.222	1.41	2.00	4.66	0.314	2.00	3.62	6.09

\* Protein calculated as  $N \times 6.37$

See also Lowenfeld *et al.*



During the first few days of lactation, human milk exhibits considerable variations in composition (see tables on pp 414 and 415) After the first week the results are more uniform In clinical work, therefore, analyses should not be made until after the sixth or seventh day of lactation Gardner and Fox, from an extensive review of the literature, find that the range of variation in the composition of healthy mothers' milks is very wide They summarise their findings as shown on p 414

After the first week of lactation the composition of human milk in round numbers may be represented as follows —

Lactose	5.5 to 8 per cent
Protein	1 to 2 per cent
Fat	2 to 5 per cent
Salts	0.1 to 0.4 per cent
Calcium as CaO	0.03 to 0.06 per cent
Total solids	10 to 15 per cent
Ash	0.1 to 0.4 per cent
Reaction	Amphoteric to litmus (pH 6.7 to 6.8)
Specific gravity	1.026 to 1.036

The fat is the most variable of the constituents During the first few days of lactation the protein decreases, whereas the lactose percentage increases From Roberts's analyses of the milks of eighty six women, Myers calculates that the average calorific value of human milk is 19 Cals per ounce (18.95 Cals)

### COMPOSITION OF COW'S MILK

The composition of cow's milk naturally varies with the breed of cow, the type of feeding, the duration of lactation, and other factors, but most analyses fall within the following limits, though the extremes of the published data are much wider (see books quoted at the beginning of this chapter)

Lactose	3.5 to 5 per cent
Protein	2.5 to 4 per cent
Fat	3 to 5 per cent
Salts	0.6 to 0.9 per cent
Calcium as CaO	About 0.15 to 0.20 per cent
Total solids	11.5 to 16 per cent
Ash	0.6 to 0.9 per cent
Reaction	Amphoteric to litmus (pH 6.7 to 6.8)
Specific gravity	1.028 to 1.035
Average calorific value, about 20 Cals per oz	

### THE DIFFERENCE BETWEEN HUMAN AND COW'S MILK

Human milk contains more lactose, less protein, less salts and less calcium than cow's milk. Moreover, there is about as much lactalbumin as caseinogen in human milk, whereas the caseinogen greatly exceeds the lactalbumin in cow's milk Heineman, in his

book, cites a table from Leach, from which the following figures are taken —

	HUMAN MILK			COW'S MILK		
	Av	Min.	Max	Av	Min.	Max.
Casemogen per cent	1 03	0 18	1 96	3 02	1 79	6 29
Lactalbumin „ „	1 26	0 32	2 36	0 53	0 25	1 44
Lactoglobulin „ „	Traces			Traces (about 0 15)		

For this reason even though cow's milk be diluted so that its total protein content is the same as that of human milk (and the requisite amount of lactose and of fat (cream) added), it cannot be made strictly equivalent to human milk. The casemogen of human milk is less readily precipitated by acid and less readily coagulated by gastric rennin, and the casein curds of human milk are smaller, looser and less flocculent than those of cow's milk, and therefore probably more readily digested.

### NOTES ON TECHNIQUE

The *total solids* are easily determined by drying a known weight (2 to 5 gm), or volume of milk thoroughly and weighing the dry residue. The *ash* may then be obtained by heating the dry solids over a very low flame until a white or pale grey ash is secured, and weighing. *Lactose* is estimated by precipitating the proteins, and then determining the lactose in an aliquot part of the protein free filtrate by applying one of the sugar methods used in urine or blood analysis. *Total nitrogen* is generally determined by a Kjeldahl or micro Kjeldahl method and from this the *protein* is calculated, using the factor  $\text{protein} = \text{N} \times 6.37$ . For *fat* one of the centrifugal methods (Gerber's or Babcock's) is generally employed owing to their simplicity and rapidity, though the Soxhlet method (Adam's paper coil method) is regarded as the most accurate. For full details of the different methods and their innumerable modifications the reader is referred to larger works and to the special manuals on milk analysis. The same sources of information should be consulted for methods of detecting *preservatives* and *adulteration*. Graham and Kay have introduced the *phosphatase* test for detecting whether milk has been pasteurised properly.

### Fat

**Babcock's Centrifugal Method** Organic matter other than fat is destroyed by sulphuric acid. The fat is separated by centrifuging and its volume is measured.

In a Babcock tube (Fig. 72) place 5 c.c. of milk. Add about 1 c.c. of concentrated sulphuric acid with a teat pipette. Mix well.

and quickly by rotation. Without delay, so that the tube shall not cool appreciably, add another portion of the acid and mix again. Continue with further portions and mixings until the fluid reaches to the bottom of the neck of the tube. At this stage the contents are very hot and black owing to charring, and the white lumps of protein which at first appeared should now have gone into solution. Allow to cool and make good the reduction in volume by again adding concentrated sulphuric acid to the bottom of the neck of the tube. Then fill to the zero mark with a mixture of equal parts of amyl alcohol and of concentrated hydrochloric acid, but do not now

mix. Centrifuge for about five minutes. If the fluid does not reach the zero mark, add water till it does, and centrifuge again. Note the percentage of fat, which is given directly by the figure on the neck of the tube, at the level of the bottom of the column of separated fat, should the upper level of the fat column again not reach the zero mark, note its reading too and make due allowance.

Sometimes the layer of fat solidifies rendering the reading difficult, in that case take a preliminary reading, and then warm the neck of the tube *carefully* in the flame until the fat just melts, and read again. If this fails to yield a well defined layer of separated fat the test has not been properly carried out and must be repeated.

The calibration of the tube can easily be checked, for 5 per cent of 5 c.c. is 0.25 c.c., and therefore the volume between the 0 and the 5 mark should be 0.25 c.c. Some of the tubes on the market are very inaccurate.

If the milk is rich in fat (over 5 per cent), use 2 or 3 c.c. of milk and 3 or 2 c.c. of water, instead of 5 c.c. of milk, and allow for the dilution in the calculation (i.e., multiply the observed fat percentage by 5/2 or 5/3 respectively). Indeed, it is wise to do this for most samples of human milk if a good

FIG 72 Babcock tube

separation of the fat is to be obtained.

The mixed amyl alcohol and HCl facilitates the separation of the fat. The amyl alcohol eventually goes into solution and does not form a separate layer.

### Lactose

In a 50 c.c. volumetric flask about half full of distilled water, place 5 c.c. of milk, 2.5 c.c. of 10 per cent sodium tungstate ( $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ ) and 2.5 c.c. of 2/3 N sulphuric acid (cf. precipitation of plasma proteins by tungstic acid at the beginning of Chapter XIX). Shake gently and add water to the 50 c.c. mark. Stopper, shake

thoroughly, and filter through any good quality paper; the filtrate should be quite clear.

The dilution of the milk is, 1 in 10. If 5 c.c. of milk are not available, lesser amounts may be used, the reagents being added in the above proportion, and the dilution being made in a measuring cylinder (e.g., 4 c.c. of milk, 2 c.c. of sodium tungstate, 2 c.c. of  $\frac{2}{3}$  N.  $\text{H}_2\text{SO}_4$ , and 32 c.c. of water).

Titrate 25 c.c. of boiling Benedict's quantitative solution (Chapter VI) with the protein-free filtrate which is placed in the burette.

*Example of Calculation.* The titration was 10.4 c.c., which correspond to 1.04 c.c. of undiluted milk. Since 25 c.c. of Benedict's solution are reduced by 0.067 gm. of lactose, therefore 1.04 c.c. of the original milk must have contained 0.067 gm. of lactose. That is, the lactose equals  $\frac{0.067 \times 100}{1.04}$ , or 6.4 gm. per 100 c.c.

### Total Nitrogen and Protein

The total nitrogen is estimated by *Kjeldahl's method*, details of which are given in the manuals of practical physiological chemistry. Five c.c. of milk are generally used, and may be digested with 10 c.c. of concentrated  $\text{H}_2\text{SO}_4$ , plus a crystal of copper sulphate (or  $\frac{1}{2}$  c.c. of a saturated solution), and 5 to 10 gm. of potassium sulphate. The ammonia, liberated by an excess of caustic soda solution, is caught in an excess of standard sulphuric acid (20 c.c. of 0.2 N.  $\text{H}_2\text{SO}_4$  or its equivalent), the excess of acid being titrated with standard sodium hydroxide solution in the usual way.

Alternatively, the *micro-Kjeldahl method* described in Chapter XIX may be utilised. The milk is diluted 1 in 10 with distilled water. Five c.c. of the diluted milk are digested as described in Chapter XIX, under "Plasma Proteins." To catch the ammonia, 25 c.c. of 0.01 N. sulphuric acid should be placed in the receiver.

The protein is calculated by multiplying the percentage of total nitrogen by 6.37; the non-protein nitrogen is negligible.

A reasonably close estimate of the proteins of milk may be secured by the Biuret Method, using 0.2 c.c. as for serum (p. 375).

Contrary to expectation the fat gives no trouble & much of it is removed with the supernatant fluid after precipitation of the proteins by trichloroacetic acid, and the rest is carried down with the precipitate of copper hydroxide in the final centrifuging. The biuret equivalent of casein(ogen) is the same as that of serum proteins. For clinical purposes the biuret method is undoubtedly satisfactory, and is much simpler than the Kjeldahl methods.

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## CHAPTER XXII

### GASTRIC ANALYSIS

**Books.** Rehfuss' *The Diagnosis and Treatment of Diseases of the Stomach*

Baessler's *Diseases of the Stomach and Upper Alimentary Tract*

Lusterman and Balfour's *The Stomach and Duodenum*

Robertson's *Gastric Acidity*

Ryle's *Gastric Function in Health and Disease*

Beaumont and Dodds' *Recent Advances in Medicine* Chapter on "The Stomach"

Hawk and Bergheim's *Practical Physiological Chemistry* Chapter on "Gastric Analysis"

THE gastric contents are examined as an extension of the clinical examination of patients suspected to be suffering from diseases of the stomach. Disorders of other portions of the alimentary tract often affect gastric secretion, causing "reflex dyspepsia," e.g., cholelithiasis, appendicitis and so on. Lastly any severe general disease may depress gastric secretion. In short, in interpreting results of gastric analysis there are three great groups of factors to consider, viz., local, reflex and general. In pernicious anaemia gastric analysis is essential to complete the diagnosis, for achlorhydria is always found in this disease and persists after successful liver treatment.

In any patient suspected to have ulceration (gastric, duodenal or malignant), chemical examination of the faeces for occult blood (see Chapter XXIV) is important both in diagnosis and in watching progress under treatment.

#### THE TEST-MEAL HISTAMINE AS STIMULUS

The Ewald test meal of toast and tea is usually employed in the single hour method. This consists of 35 gm ( $1\frac{1}{4}$  oz) of toast without butter, and 250 c.c. ( $\frac{1}{2}$  pint) of tea without milk. Unfortunately a number of variations in the quantity of toast and of tea have been made, some physicians allowing a pint of tea and more than the above quantity of toast, and others allowing butter or milk or both. The meal has therefore not been strictly "standard." Though no exact experiments appear to have been made to decide the point, the probability is that these variations in the test meal have not led to any serious difficulty in interpreting the results of analyses.

The two chief objections to the Ewald meal are that the dextrin from the toast may impart a pale brown tint to the gastric contents, thereby complicating the naked eye detection of bile or blood in small amounts, and lumps of partially digested toast may block the tube.

The Bors test-meal of oatmeal gruel was designed to overcome these difficulties. The gruel is practically colourless and of such a consistency as not to block the tube. It is prepared by boiling 2 tablespoons of breakfast oatmeal with a quart of water down to a pint, straining through muslin, and adding sugar to taste. Salt should not be added, because chloride estimations would thereby be rendered valueless. *Milk must not be given* with the gruel or as a separate drink, because, owing to its buffer action, it reduces greatly the free HCl, and may thus lead to gross errors in interpretation. The analyst will detect the mistake by the milky appearance, and also by the big difference between the total acidity and free HCl results for each sample (commonly some 40 to 50 instead of the usual 10 to 15 c.c. N/10 per cent.)

Alcohol, either 50 or 100 c.c. of a 7 per cent. solution in water, has also been extensively used as a test meal (*cf.* Bloomfield and Keefer). From the analyst's point of view its great advantage is that the gastric samples are cleaner and more easily cleared. Some workers, however, do not regard it as so sure a stimulus to gastric secretion as gruel.

In experimental work gastric analyses are performed after all sorts of meals, *e.g.* when determining the emptying rate after different foods, but in clinical work it is necessary to adopt a standard meal in order to compare the pathological with the normal under definite conditions. The above are the three test meals most commonly employed, though others have been recommended from time to time, *e.g.*, water (Bergeim *et al.*), caffeine (*cf.* Becker and Thaler), etc. In infants and young children, however, it is rarely practicable to give a standard test meal. At that age gastric analysis is commonly made after a known quantity of milk or after one of the ordinary feeds.

Subcutaneous injections of histamine—0.25 to 1 mgm. of the hydrochloride—are often used in the investigation of cases of achlorhydria. In true achylia gastrica the injection does not cause a secretion of acid, *e.g.*, in pernicious anaemia, but in most cases of other forms of achlorhydria, *e.g.*, due to neutralisation of HCl by regurgitation, to psychical or reflex causes, or to sprue (Fairley), the histamine often does cause a secretion of HCl.

It is a common practice to collect the first three or four gastric fractions, then to give the injection three quarters to one hour after the gruel, and thereafter to continue the collection of the gastric samples as usual in the fractional method, there is, of course, no point in giving the histamine if the early samples do contain free HCl.

Another method (*cf.* Lander and MacLagan) is to use histamine as the sole stimulus, in other words as the 'test meal'. This is an abnormal stimulus, and caution is advised if the blood pressure, which should always be taken before the injection, is below 110 mm. Hg. Further experience is required before its clinical value can be judged properly, but it has the advantage that the volume of juice and of HCl secreted per unit of time can be measured and compared

with the range in health, a practical difficulty is uncertainty that the gastric contents have been removed completely, and no knowledge of how much, if any, of the secretion has escaped through the pylorus, or of the volume of regurgitated fluid

### TYPES OF STOMACH TUBE

In infants and young children a small œsophageal tube and syringe are commonly employed (see Fig 73). In adults the Ewald test meal is generally removed by an œsophageal tube of larger size (up to size 24) or by a piece of rubber tubing which is fairly rigid and has a bore of about 6 or 7 mm—the ordinary “stomach tube”. In the fractional test meal a smaller and more flexible tube is used, with some form of metal olive attached to one end

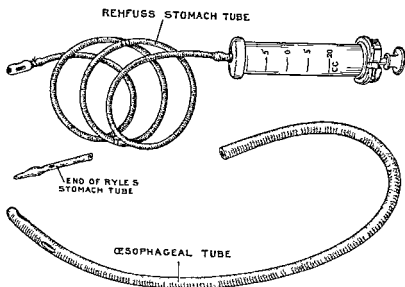


Fig 73 Tubes for removal of gastric contents

and a syringe at the other. In the original pattern devised by Einhorn, the holes in the metal olive were too small. Numerous modifications of the olive have been introduced by Rehfuß and others. In the Rehfuß tube, illustrated in Fig 73, the holes in the metal olive are of the same bore as that of the rubber tube. In Ryle's modification (see Fig 73) there is a terminal piece of metal coated with rubber to prevent damage to the mucous membrane. The lump of metal makes the tube easier to swallow, and is easily seen on the radiographic screen in experiments in which it is necessary to locate the position of the end of the tube. In this pattern there are holes in the side of the tubing just above the enclosed lump of metal. The writer prefers the Rehfuß pattern because it is less likely to become blocked than Ryle's tube, but with the latter there is less likelihood of traces of blood getting into the gastric samples owing to trauma.

## TECHNIQUE OF REMOVAL OF GASTRIC CONTENTS

The tube is lubricated with liquid paraffin, which does not mix with the gastric contents. Infants will swallow the tube more readily if glycerol is used as lubricant, because it is sweet, an excess of this material, however, should be avoided, because it dissolves in the gastric fluid. The tip of the tube or metal olive is placed on the back of the tongue and the patient instructed to swallow three or four times, and then to breathe vigorously through the nose.

During its passage down the first part of the throat the tube is very apt to cause retching. This can often be inhibited or lessened by repeatedly instructing the patient to breathe forcibly through the nose, gripping the tube meanwhile between the lips, not between the teeth. When the desire to retch becomes more under control another portion is pushed down the patient swallowing and separating the lips. If the patient can be persuaded to help by pushing the tube down himself bit by bit as described, retching will be less troublesome, because he is less likely than the operator to push the tube against the sensitive soft palate, and because he will gain confidence.

The tubes are ringed round at certain levels (*e.g.*, a single ring at 16 in., two rings at 20 in., and three rings at 24 in. from the tip (see Fig. 73), in order that the operator may know how much tube he has passed. In practice these rings are a useful rough guide but their position should not be regarded as providing certain evidence that the tube is in the stomach. In pathological cases the distance from the teeth to the bottom of the stomach varies considerably. The tube should be passed to between the first and second rings and aspiration made. If no fluid is obtained the patient is instructed to swallow again and aspiration is repeated when the lips lie between the second and third mark, and so on. Occasionally as much as 30 in. of tube must be swallowed before the gastric contents are reached. The tube being in position, aspiration is made with a 20 c.c. syringe, the piston of which is lubricated with liquid paraffin.

The above is a general description of the passage of a flexible stomach tube, whether the resting juice, one hour sample or fractional samples are to be obtained. When a more rigid tube is employed the gastric contents may syphon over without any suction, or the flow may be started by asking the patient to strain. Instead of a syringe, some form of aspirating bottle (*e.g.*, a Senoran's bottle) may be attached.

Whichever method be utilised there are two essentials to be observed. (1) The patient should be supplied with a sputum mug, or beaker, or any convenient vessel into which he is instructed to expectorate in the intervals while the tube is being passed and while it remains in the stomach. *He must not swallow his saliva.* The importance of this will be obvious if it is realised that some patients may secrete several hundred cubic centimetres of saliva during the course of a fractional test meal. (2) *Water must not be used to facilitate the removal of the gastric contents.* The use of water



and of other fluids is, of course, quite permissible when washing out the stomach for therapeutic purposes, but a "stomach wash out" is useless for analysis. In removing a test meal no fluid must be added at all, because it would introduce an unknown dilution. Even if the volume of fluid introduced were measured the result would be hopeless, because it would be impossible to estimate the fraction that would escape through the pylorus. In the fractional method, after the withdrawal of each sample, air should be forced down the tube to clear it. Otherwise the first 1 or 2 c.c. aspirated the next time may consist of the previous sample.

When withdrawing the tube the patient is instructed to open his mouth and depress his chin on his chest, but not to throw back his head.

## METHODS OF EXAMINATION

### Vomit

Fasting juice or gastric residuum

One hour test meal (Ewald)

Fractional test meal

Alveolar  $\text{CO}_2$  curve after test meal

### Examination of Vomit

The character of the vomiting, whether simple or projectile and so on should be noted. The amount and naked eye appearance of the vomit is observed and any queries raised by these examinations are followed up. As a general rule, however, chemical examination of the vomit gives little information. Testing for blood is probably the most important. A simple method for identifying blood in 'coffee grounds' vomit with the aid of sulphuric acid and a spectroscope is given at the end of this chapter. It does not take a minute to test the reaction with litmus, but quantitative analysis is generally useless because the gastric secretion is usually mixed with unknown amounts of food in varying stages of digestion, and often with unknown quantities of duodenal contents. For this reason bile is frequently present. In special cases, or if a thorough examination should be requested, the chemical investigation follows the same lines as laid down for the one hour test sample.

### Examination of the Gastric Residuum

A charcoal biscuit is given the night before. Normally this will have all passed through the pylorus by the morning.

The gastric contents are removed before breakfast. A flexible tube is used and the stomach is emptied completely by evacuating first in the sitting position, then in turn with the patient lying on his back, on his left side, on his stomach, and on his right side, and finally in the sitting position again.

The volume in cubic centimetres, the naked-eye appearance, and the presence or absence of charcoal are noted. The unfiltered fluid is examined as to its reaction, and for bile, blood, starch and mucus. The rest of the fluid is cleared by filtering or centrifuging,

and is then examined qualitatively for free HCl and lactic acid and quantitatively for free HCl and total acidity. In special cases (or as a routine if facilities admit) the filtered fluid is also examined quantitatively for mineral and total chlorides and for pepsin. Lastly some of the deposit is examined microscopically.

The normal findings are given below

*Normal Fasting Juice*

Charcoal	Absent
Volume c c	20 to 150 usually 20 to 100
Reaction to litmus	Generally acid
Free HCl { c c N/10 per cent gm per cent	0 to 70 usually 0 to 30 0.00 to 0.26 usually 0 to 0.11
Total acidity { c c N/10 per cent gm per cent as HCl	2 to 80 usually 10 to 50 0.01 to 0.29 usually 0.04 to 0.18
Lactic acid	Nil
Blood	Absent or traces due to trauma by tube
Bile pigment	Present or absent
Starch	Nil.
Mucus	Traces only
Pepsin index (Mett)	0.7 to 5.0
Freezing point °C	- 0.816 to - 0.298 (Rehfuß)
Specific gravity	1.010 to 1.0036 (Rehfuß)
Trypsin index	0 to 16 (Rehfuß)
<i>Microscopical Examination</i>	
Leucocytes	Few
Erythrocytes	Nil or few due to trauma by tube
Epithelial cells (squamous excepted)	Nil
Gross food residues	Nil
Vegetable debris	Little occasionally
Meat fibres	Nil

An examination of the gastric residuum is the simplest way of examining gastric function and frequently gives as much information as the more elaborate Ewald test method or fractional test meal. Its disadvantage is that it gives little idea of what would be the response of the stomach to the stimulus of food. The finding of 'hyperchlorhydria' (over 60 c c N/10 per cent)

however, may be important either in diagnosis (*e.g.*, in ulcer) or as a guide to treatment with alkali. The great advantage of examining the fasting juice is that the microscopical picture is not complicated (and diluted) by the presence of food. It is therefore particularly useful in carcinoma. Indeed, in this condition the foul smelling residuum, with its bulky deposit containing charcoal gross food residues, many leucocytes, altered blood and organic acids, may be almost pathognomonic. Very rarely tumour masses or tumour cells may lead to a certain diagnosis. An excessive volume (over 150 c.c., up to 500 c.c. or more) of fasting contents points to atony of the stomach, simple or malignant. The significance of blood in traces is often difficult to assess. It may occur through trauma by the tube, bleeding from the gums, or oozing from the mucous membrane as in pernicious anæmia, or from dilated œsophageal veins as in cirrhosis of the liver, or it may represent "true hæmorrhage" from an ulcer, whether innocent or malignant. In rare cases the passage of the tube may be obstructed in the œsophagus, at which point aspiration may draw a little blood. Such an observation points to carcinoma of the œsophagus, and no further attempt should be made to pass the tube. The presence of altered blood is generally more significant than streaks of fresh blood, but if there is free HCl in the stomach oxyhæmoglobin is "altered" in a few seconds to acid hæmatin. The presence or absence of bile is of little significance, since either may occur normally. The presence of starch simply means that food has not passed through the pylorus properly, either on account of spasm of that sphincter or of dilatation and atony of the stomach. The chemical test for starch is therefore a test for one of the food residues. If the swallowing of saliva is prevented, mucus should normally be present only in traces. It may be found in excess in mucous gastritis, but is not commonly in such obvious excess as to be of any significance.

### The One-Hour Test-Meal (Ewald)

The patient, after a night's fast, is given a test breakfast, which is removed at the end of an hour. The volume and naked eye appearance are noted, and the same chemical tests as listed under the examination of the gastric residuum are applied.

Lactic acid is due to fermentation (see Dodds and Robertson), and is formed only when free HCl is absent or low, less than 20 c.c. N/10 per cent, because free HCl inhibits or stops the action of lactic acid forming organisms. Qualitative tests for the organic acid are positive in many cases of advanced gastric carcinoma, but positive results are found also in non malignant pyloric obstruction with stagnation of gastric contents. The ranges of variations obtained in health and in certain diseases for free HCl, total acidity, total and mineral chlorides, active HCl, etc., are given in the tables on pp. 427 and 428.

One hour Test meal Chemical Findings

	Normal			Ulcer				Carcinoma				Chronic dyspepsia and/or gastritis				Pernicious anemia			
	c c N/10 per cent		gm % as HCl	Gastric		Duodenal		c c N/10 per cent		gm % as HCl		cc N/10 per cent		gm % as HCl		c c N/10 per cent		gm % as HCl	
	Min	Max	Min	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
Free HCl	0	70	0.00	0	0.00	0	95	0	95	0.00	0.35	0	70	0.00	0.25	0	0	0.00	0.00
Total acid	5	100	0.02	10	100	0.04	0.36	15	110	0.05	0.40	3	80	0.01	0.22	0	40	0.00	0.15
Total cl forides	55	110	0.20	50	120	0.18	0.44	70	130	0.25	0.48	20	110	0.08	0.40	45	90	0.17	0.33
Mineral chlorides	20	50	0.07	10	55	0.04	0.10	10	65	0.04	0.24	10	65	0.04	0.24	15	65	0.05	0.24
Active acid	5	100	0.00	10	95	0.04	0.36	15	110	0.05	0.40	3	80	0.01	0.09	0	40	0.00	0.15
Remarks	About 4 % normal people have achlorhydria about 10 % hyperchlorhydria			No characteristic acid or chloride findings		Commonly hyperchlorhydria hyperacidity and high total chlorides		Commonly achlorhydria hyposecrecity and low total chlorides		Commonly achlorhydria hyposecrecity and low total chlorides		No characteristic acid or chloride findings		100 % achlorhydria. Commonly low acid and total chloride figures					

\* Achlorhydria increases with age (see p 428)

These tables have been compiled after a fairly exhaustive search of the literature and from the writer's own results. They could be considerably extended (*cf* statistics of Vanzant *et al* in health and in ulcer, and of Comfort and Vanzant in carcinoma), but suffice to show that, with the exception of pernicious anaemia, almost any result may be obtained in almost any disease. In other words, in dealing with a given patient, gastric analysis will very rarely enable a diagnosis of any one disease to be made with certainty. That does not mean that all these tests are valueless. In conjunction with the clinical examination they often give information useful in arriving at a diagnosis, and are valuable when repeated at intervals in order to follow the results of treatment intended to alter the reaction of the gastric contents. Thus a diagnosis of pernicious anaemia is unlikely to be correct if there is not achlorhydria. Hyperchlorhydria is a point in favour of duodenal ulcer, but its absence does not exclude such a diagnosis. Most cases of carcinoma, according to some authorities, *e.g.*, Friedenwald, 90 per cent or more, have achlorhydria or hypochlorhydria, and so on. On the other hand, the accumulated results tend to show that gastric analysis is of little help in most cases of gastric ulcer and of gastritis, because there is no one group of findings common to a majority of these cases.

*One hour Test-meal—(continued)*

(cont'd) Chemical and microscopical *	Normal	Ulcer		Carcinoma	Chronic dyspepsia and/or gastritis	Pernicious anaemia
		Gastric	Duodenal			
Lactic acid	0	0	0	0 or +	0 or +	0 or +
Blood	0 or + (trauma)	0 or +	0 or +	0 or +	0 or +	Often +
Bile	0 or +	0 or +	Usually 0	0 or +	0 or +	0 or +
Volume c.c.	20 to 100	40 to 150	20 to 110	10 to 500	20 to 200	5 to 50
W b c	+ (saliva)	+ to +++	0 to +	+ to +++	+ to +++	+ to +++
R b c	0 or + (trauma)	0 to +++	0 to +	0 to +++	0 to +	+ to +++
Ep c (excluding squamous ep c)	0	+ to +++	0 to +	+ to +++	+ to ++	+ to ++
Tumour cells	0	0	0	0 or +	0	0
Sarcinae	0	0 to +	0	0 to +++ Usually 0	0 to ++	0
Yeast (from meal)	+	+	+	+	+	+
Boss-Oppler B	0	0	0	0 to +++	0 to +	0
Starch (from meal)	+	+	+	+	+	+
Cellulose remains	0	0 to +	0	0 to +++	0 to +++	0

\* Oily drops from the lubricant will be seen

Vanzant *et al* showed that in health achlorhydria became more frequent with increase of age, in the twenties about four, and in the sixties about twenty five out of every 100 individuals had no free HCl.

An objection to the one hour test method is that in certain pathological conditions the volume of fluid in the stomach *before* the meal may be large, and may modify the findings in the one hour sample. In other words, in these cases a mixture of residuum and the hour's secretion plus the partially digested food, less that portion of this mixture which has passed through the pylorus (plus regurgitated fluid from the duodenum, if any) is examined. This objection may be met by first removing the residuum, leaving the tube *in situ*, giving the meal, and at the end of an hour removing the gastric contents. Other possible combinations of the examination of the gastric residuum plus examination(s) subsequent to the meal will occur to the reader, but the most standardised procedure is the 'fractional test method'.

In the one hour test sample microscopical examination is rarely of any value, because the vast bulk of the deposit consists of starch granules and yeast cells from the toast or of imperfectly digested gruel.

### The Fractional Method of Examination

The residuum is removed, the tube left *in situ*, and the oatmeal gruel swallowed, the time of beginning the meal being noted. Samples of 5 to 10 cc are withdrawn every fifteen or twenty minutes until the stomach is empty, usually a matter of two or three hours. The end of the tube is clipped on to the coat in the intervals between the collections. Each sample is labelled. The residuum is examined as previously described. Each subsequent sample is examined qualitatively for blood, bile, starch and mucus, and quantitatively for free HCl, total acidity, and in special cases for total and mineral chlorides. The quantitative findings may be plotted as curves.

The difficulties in assessing the significance of traces of blood have already been discussed. If, however, blood persists in several of the fractional samples, the hæmorrhage is more likely to be pathological, and not accidental due to trauma by the tube. Bile, though often present in a normal residuum, does not normally appear in the subsequent samples until the stomach is almost empty. In pathological cases it may appear much earlier on the curve, showing that the pylorus is patent or has relaxed prematurely. It is a matter of controversy whether in normal individuals regurgitation of duodenal juice not containing bile does or does not occur. From the physiological standpoint the modern view is that mucus plays a more important part than regurgitated fluid in controlling normal gastric acidity (see papers in *J. Physiol.* by MacLennan and Griffiths, Apperly and Norris, Bolton and Goodhart, from 1928 to 1936, consult also references to Lander and MacLennan, Welin and Frisk at the end of this chapter).

It is an undisputed fact that in pathological cases such regurgitation of duodenal fluid does occur, because bile not uncommonly appears in early fractions. Examination for bile is, therefore, important in that it gives information as to the state of

the pylorus. As previously stated, the presence or absence of mucus is not usually of much significance, though Hurst lays great stress on its presence as evidence of gastritis. The test for starch is said to enable us to measure the emptying rate of the stomach. By this test the stomach in healthy individuals empties in from one to two and three quarter hours, with an average of two hours. In the writer's opinion however, further investigation is required, because he has often found the iodine test for starch positive in all samples right up to the point when no more fluid could be aspirated. If it be true that starch is often present till the stomach is empty, the iodine test is obviously frequently superfluous. The difference in observations may be accounted for in part by the method of testing for starch. If the dilution of the starch is great it will not be detected unless very dilute iodine is added drop by drop. *A priori*, though it is recognised that gastric secretion is continuous, it would be expected that little or no fluid could be aspirated fifteen minutes after all food has passed through the pylorus unless there is hypersecretion. No doubt if aspiration was again repeated after an additional fifteen, thirty or sixty minutes, gastric secretion would finally be recovered from every subject which was free from starch, but in practice the tube is rarely retained for longer than a total of three hours on account of the discomfort to the patient. However, some workers only attach significance to gross clumps of starch, and in practice the amount of sediment in the different fractions is probably the best guide to the rate of emptying.

The quantitative examination on which most work has been done is that for free HCl. Bell has made the following classification:—

Achlorhydria. Never any free HCl

Hypochlorhydria. Free HCl never above 10 c c N/10 per cent

Hyperchlorhydria. One or more points on the curve above 60 c c N/10 per cent

On this basis he has classified the results on 100 normal students examined by Bennett and Ryle, and his own series of pathological cases. These findings, together with a number of others from the literature, including those of Moynihan and of Wills, using the Boas meal, are collected in the table on p. 431.

The numbers in each group are too small to be of value statistically, but it will be seen at a glance that in pernicious anaemia only is there a constant finding, viz., achlorhydria, moreover, as previously noted, this persists after an injection of histamine and after successful treatment by anti anaemia factor (liver, etc.). From the clinical point of view in this disease all that is necessary is to collect the fractional samples and to test each qualitatively for free HCl. If free HCl is present, then the diagnosis of pernicious anaemia is open to serious doubt. In the other diseases the same remarks apply for the fractional method as for the Ewald meal. In most cases of carcinoma of the stomach there is achlorhydria or hypo-

chlorhydria and in the majority of cases of duodenal ulcer there is hyperchlorhydria. At the same time a small proportion of healthy individuals show either achlorhydria or hyperchlorhydria, so the acid findings can never be pathognomonic.

*Results of Fractional Test-meals*

Condition	No of Cases	Achlorhydria		Hypochlorhydria		Isochlorhydria or Normal		Hyperchlorhydria	
		No	Per cent	No	Per cent	No	Per cent	No	Per cent
Normal	100	4	4.0	1	1.0	87	87.0	8	8.0
Gastric ulcer	84	6	7.1	10	11.9	51	60.7	17	20.2
Duodenal ulcer	135	4	3.0	4	3.0	59	43.7	68	50.3
Carcinoma of stomach	22	9	40.8	6	27.3	6	27.3	1	4.6
Chronic gastritis	11	5	45.4	2	18.2	3	27.3	1	9.1
Nervous dyspepsia	37	2	5.4	4	10.9	16	43.2	15	40.5
Neurasthenia	20	2	10.0	6	30.0	7	35.0	5	25.0
Psychasthenia	11	1	9.1	2	18.2	8	72.7	0	0.0
Visceroptosis including gastropptosis	19	2	10.5	5	26.3	8	42.1	4	21.0
Gallstones	12	1	8.3	0	0.0	9	75.0	2	16.7
Chronic appendicitis	26	5	19.2	0	0.0	16	61.5	5	19.2
Tabes dorsalis	9	1	11.1	1	11.1	5	55.5	2	22.2
Disseminated sclerosis	8	0	0.0	1	12.5	7	87.5	0	0.0
Rheumatoid arthritis	14	5	35.7	1	7.1	5	35.7	3	21.4
Addison's anaemia	9	9	100.0	0	0.0	0	0.0	0	0.0
Secondary anaemia	6	2	33.3	0	0.0	4	66.7	0	0.0
Hyperthyroidism (Moll and Scott)	50	22	44.0	15	30.0	13	26.0	0	0.0
Rosacea (Brown)	50	7	14.0	8*	16.0	31	62.0	4†	8.0
Miscellaneous skin diseases (Brown)	50	6	12.0	5*	10.0	26	52.0	13†	26.0

\* Hypochlorhydria defined as less than 20 c.c. N/10 per cent

† Hyperchlorhydria defined as 55 c.c. N/10 per cent or more

In following the results of treatment the fractional test meal has considerable value (*cf* Hurst) and in research work it has proved a most useful weapon but from the clinical point of view it is now generally agreed that it gives very little help in diagnosis.



In the writer's opinion, owing to the considerable labour, time and expense involved, and the fact that its limitations have been established the test should now be restricted to pernicious anæmia, to a few special or difficult cases, and to research work.

The total acidity is commonly some ten to fifteen points above the free HCl (expressed as c.c. N/10 per cent). If the difference between the two estimations is greater than twenty points, it generally indicates that organic acids are present. This is observed when there is stagnation of the gastric contents, as in simple atony or carcinoma of the stomach (*cf.* also remarks on buffer action of milk, p. 421).

Chloride estimations give a better measure of the secretory power of the stomach than acid estimations, but such methods are frankly of very limited value in clinical work, though of the greatest interest in research. They are sometimes of value in differentiating achlorhydria due to regurgitation from other achlorhydrias. A case should not be labelled achylia unless it has been shown that pepsin is absent.

#### Alveolar $\text{CO}_2$ Curve after a Test-meal (Bennett and Dodds)

When hydrochloric acid is secreted into the stomach, the blood is left with a greater proportion of basic radicles, but the reaction (hydrogen ion concentration) does not alter, because these basic radicles are combined with  $\text{CO}_2$  and the tension of carbonic acid in the blood increases. As a result the tension of alveolar  $\text{CO}_2$  likewise increases (see Fig. 74). Measurements of the alveolar  $\text{CO}_2$  before and at intervals after a test meal give a curve which runs parallel with the total secretion of HCl (though not with the curves of free HCl or total acidity).

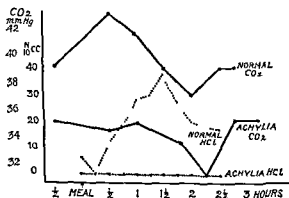


FIG. 74. Curves of alveolar  $\text{CO}_2$  tension and of gastric free HCl in two healthy subjects (one of whom was achlorhydric) following test meals (After Bennett and Dodds).

At first sight this method should be useful in clinical work, in that it would be less unpleasant for the patient than the passage of a stomach tube. Unfortunately there are two factors which greatly limit its routine use. In the first place, it is not easy in many

patients to secure samples of true alveolar air repeatedly, and, secondly, very accurate gas analysis is essential

## THE VALUE OF GASTRIC ANALYSIS IN CLINICAL WORK

This has already been indicated in previous paragraphs but is summarised below

The laboratory report is never pathognomonic of any one disease. The results can only be properly interpreted with a knowledge of the clinical findings. The chief difficulty regarding the quantitative findings for acid and chlorides is that the range of variations in healthy individuals is nearly as wide as the range in pathological conditions. The chief value of an examination of the gastric residuum is in cases of suspected carcinoma. A foul smelling thick resting juice with a large deposit containing charcoal from overnight food *débris* altered blood pus and organic acids is almost diagnostic but this combination of findings occurs late in the disease. Rarely tumour cells are found. The Ewald meal is most useful in carcinoma and duodenal ulcer. Most patients with malignant disease have achlorhydria or hypochlorhydria but normal acid findings and even hyperchlorhydria may occur, thus in carcinoma of the body or cardiac end of the stomach hyperchlorhydria may be found. Many patients with duodenal ulcer show hyperchlorhydria. The main utility of the fractional test meal has been summarised by Beaumont and Dodds as follows —

Examination of the pyloric function

Diagnosis of juxta pyloric ulcers and obstruction

Diagnosis of carcinoma and investigation of cases of achlorhydria

The rate of emptying and the presence or absence of bile at the different stages gives useful information regarding pyloric function. The diagnosis of obstruction depends mainly on the presence of charcoal and food residues in the resting juice and the absence of bile, the subsequent fractional analysis is therefore generally superfluous. The laboratory diagnosis of juxta pyloric ulcer depends mostly on obtaining a "climbing" type of curve (free HCl climbs steadily to a high figure) but this type of curve is also found occasionally in healthy individuals, and in other pathological lesions and it is sometimes absent in cases of juxta pyloric ulcer. The diagnosis of carcinoma depends mostly on the examination of the residuum. Achlorhydria can only be satisfactorily demonstrated by fractional analysis, but for this purpose qualitative tests for free HCl are alone essential. The writer, therefore, agrees with the subsequent remark of the authors quoted viz "The fractional test meal is essentially a clinical test almost as much can be learnt from the manner in which the specimens are withdrawn, and their naked eye appearance as from the quantitative analysis." In his experience, it has been but seldom that the labour spent on a thorough chemical examination of the fractional samples

has been of real assistance to his clinical colleagues in making a diagnosis. In research and in investigations on the effect of different lines of treatment fractional test meals have been valuable, a fact well illustrated by the series of excellent papers published by the group of workers at Guy's Hospital.

### Summary of Personal Recommendations

(1) As a routine method of investigation examine the resting juice. This will usually give all the information desired in carcinoma and in pyloric obstruction. In the vast majority of other conditions it will indicate whether test meals are likely to be of value or not.

(2) If further examination is required administer an Ewald meal. Occasionally the results will be helpful but in most instances they will not assist or will give no further information than that already obtained from the resting juice.

(3) Reserve the fractional method for (a) the demonstration of achlorhydria, as in pernicious anaemia (in which qualitative tests for free HCl are alone necessary), (b) following the results of treatment by alkali etc., in special cases (in most patients clinical observations alone are sufficient), (c) research work.

### DEFINITION OF TERMS

	One hour Method	Fractional Method
<b>Free HCl</b>		
Achlorhydria	Absent	Absent throughout
Hypochlorhydria	$\left\{ \begin{array}{l} 1 \text{ to } 19 \text{ c.c. N/10 per cent} \\ 0.001 \text{ to } 0.069 \text{ gm per cent}^* \dagger \end{array} \right.$	Never above 10 c.c. N/10 per cent ( $\approx 0.04$ gm per cent as HCl) $\dagger$
Isochlorhydria or normal free HCl.	$\left\{ \begin{array}{l} 20 \text{ to } 60 \text{ c.c. N/10 per cent} \\ 0.07 \text{ to } 0.22 \text{ gm per cent}^* \end{array} \right.$	Ranges between 11 and 60 c.c. N/10 per cent or 0.04 and 0.22 gm per cent as HCl $\dagger$
Hyperchlorhydria	$\left\{ \begin{array}{l} \text{Over } 60 \text{ c.c. N/10 per cent} \\ \text{Over } 0.22 \text{ gm per cent}^* \end{array} \right.$	One or more points above 60 c.c. N/10 per cent or 0.22 gm per cent $\dagger$
<b>Total acidity</b>		
Anacidity	Absent	Absent throughout
Hypoacidity	$\left\{ \begin{array}{l} 1 \text{ to } 29 \text{ c.c. N/10 per cent} \\ 0.001 \text{ to } 0.109 \text{ gm per cent (as HCl)}^* \end{array} \right.$	Never above 20 c.c. N/10 per cent ( $\approx 0.07$ gm per cent as HCl) $\dagger$
Isoacidity or normal total acidity	$\left\{ \begin{array}{l} 30 \text{ to } 70 \text{ c.c. N/10 per cent} \\ 0.11 \text{ to } 0.26 \text{ gm per cent (as HCl)}^* \end{array} \right.$	21 to 70 c.c. N/10 per cent or 0.08 to 0.26 gm per cent as HCl $\dagger$
Hyperacidity	$\left\{ \begin{array}{l} \text{Over } 70 \text{ c.c. N/10 per cent} \\ \text{Over } 0.26 \text{ gm per cent (as HCl)}^* \end{array} \right.$	Over 70 c.c. N/10 per cent. Over 0.26 gm per cent as HCl $\dagger$

\* The writer has been unable to trace any convention in this matter. The above is suggested.

$\dagger$  20 c.c. N/10 per cent has been selected as the boundary between hypochlorhydria and isochlorhydria because though there is no definite statement in the literature the impression received from studying the discussions in papers is to that effect.

$\dagger$  Bell's classification.

### Achylia

Absence of free HCl and of pepsin with low chloride values i.e. absence of gastric secretion.

Relationship of "active HCl" (active acidity<sup>1</sup>) to total acidity.

Total acidity (I)  $\left\{ \begin{array}{l} \text{Free HCl (II)} \\ \text{HCl combined with proteins} \\ \text{Acid salts (phosphates)} \\ \text{Organic acids} \end{array} \right\}$  "Active HCl"

Relationship of "active HCl" to chlorides.

Total chlorides (III)  $\left\{ \begin{array}{l} \text{Observed mineral chlorides (IV)} \\ \text{"Active HCl"} \end{array} \right\}$   $\left\{ \begin{array}{l} \text{Free HCl} \\ \text{HCl combined with proteins} \\ \text{Acid salts (phosphates)} \end{array} \right\}$

If total acidity (I), free HCl (II), total chlorides (III), and mineral chlorides (IV) are estimated, and each is expressed in the same terms, either as c c N/10 per cent, or as gm per cent calculated as HCl, we can calculate the following by difference —

"Active HCl" (active acidity<sup>1</sup>) from total chlorides (III) minus mineral chlorides (IV)

Organic acids, from total acidity (I) less "active HCl" ( (III) minus (IV) )

HCl combined with proteins plus acid salts, from "active HCl" ( (III) minus (IV) ) less free HCl (II)

If there are no organic acids the total acidity will be the same as the "active HCl" (total less mineral chlorides) If the active HCl (total less mineral chlorides) is appreciably greater than the total acidity there is something wrong in the analysis or in the calculations The following are examples of such calculations —

	Fluid A	Fluid B
II Free HCl, c c N/10 per cent	43	0
I Total acidity, c c N/10 per cent	55	46
III Total chloride, c c N/10 per cent	79	52
IV Mineral chloride c c N/10 per cent	25	38
III-IV "Active HCl," c c N/10 per cent	54	14
I-(III-IV) Organic acids, c c N/10 per cent	1	32
(III-IV)-II HCl combined with proteins (plus acid salts) c c N/10 per cent	11	14

### TECHNIQUE OF THE SIMPLER TESTS

The unfiltered juice is used for testing the reaction, and for free HCl (qualitatively only), blood and starch The deposit (centrifuged if necessary) is examined microscopically Tests for bile should be made on the precipitate or the filtrate, or both, when there is the least suspicion of a yellow tinge For quantitative estimation of free HCl, total acidity, total and mineral chlorides, the filtrate is used Tests for lactic acid are likewise performed on the filtrate

### Methods of Clearing Gastric Contents

Simple filtration through paper is generally slow, but usually suffices when examining the gastric residuum or the one hour

<sup>1</sup> When ashing gastric contents for the estimation of mineral chlorides free HCl and HCl combined with proteins is volatilised, but furthermore acid phosphates interact with neutral chloride to yield neutral phosphates and HCl which is driven off That is why acid salts are included in "active HCl" which would be better described as "active acid" The acid salts, however, are present in traces only, and in practice may be disregarded

sample Centrifuging is much quicker, but it is often difficult to obtain a satisfactory separation of mucus, which tends to rise towards the top. Centrifuging plus filtration is frequently satisfactory. Another device which was shown the writer by one of Sir F. Gowland Hopkins' pupils is illustrated in Fig. 75. This method is particularly valuable in clearing the small samples obtained in the fractional method but requires a little practice. The test tube should have a stout wall, and the wad of absorbent cotton wool must be very tightly packed and about an inch thick. The wad is pushed down with the glass rod till it reaches the surface of the fluid; a pause is made till the wool is wetted a little, and then

the wad is slowly pushed through the liquid. The fluid which passes through and lies above the wool is generally clear. A syringe (B in Fig. 75) may be used in the same way, and is more convenient. It is advisable to lubricate the piston with liquid paraffin. To remove the plug from the barrel, the piston is pulled out sharply, when the plug will follow about half way up the barrel, when it is easily removed with forceps. The simplest and most satisfactory method of all (C in Fig. 75) is to put a fairly tight plug of wool above the fluid in a non-tapering centrifuge tube and then to centrifuge at high speed for fifteen minutes or longer until the wool has been driven down, carrying with it all mucus (D in Fig. 75).

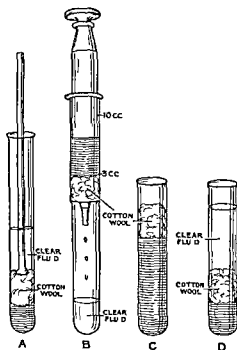


FIG. 75 Methods of clearing gastric contents

Fluids containing large amounts of mucus (usually from neglect to instruct the patient to avoid swallowing saliva) are unsatisfactory for quantitative analyses. The writer has found in such specimens that big variations may result solely from sampling. Thus the mineral chlorides in one portion containing little mucus may appear higher than the total chlorides in another portion containing much mucus—an absurd finding.

### The Individual Tests

Note odour, colour (bile, blood), consistency (mucus), and sediment

Test reaction to litmus

Free HCl (qualitative)

(a) *Thymol Blue* To  $\frac{1}{2}$  in column of the gastric fluid in a test-

tube add a few drops of 0.1 per cent thymol blue solution. If a red colour develops free HCl is present.

(b) *Gunzburg's Test* Preparation of reagent. Mix 6 drops of 10 per cent phloroglucin in absolute alcohol with 3 drops of 10 per cent vanillin in absolute alcohol.

In a porcelain evaporating basin place 1 drop of the gastric fluid and immediately after 1 or 2 drops of Gunzburg's reagent and mix at once. Put the basin on a boiling water bath and allow the contents to dry *completely*. A brilliant red colour indicates the presence of free HCl.

(c) *Topfer's Test* To  $\frac{1}{2}$  in. column of the gastric fluid in a test tube add a few drops of Topfer's reagent ( $\frac{1}{2}$  per cent dimethyl aminoazobenzene in 96 per cent alcohol). Free HCl is present if the mixture turns red.

Gunzburg's reagent is the most specific for mineral acid. (Hydrochloric is usually the only mineral acid present, but tests for 'free HCl' would, of course, be positive if free sulphuric was present, e.g., after swallowing this acid). Thymol blue (pH range 1.2 to 2.8) is more satisfactory than Topfer's reagent (pH range 2.9 to 4.2). The latter will give positive reactions with lactic acid in concentrated solution. Thymol blue is recommended for routine work.

### Lactic Acid

*Uffelmann's Test* In a beaker or flask mix two test tubes full of distilled water, one test tube full of 5 per cent carbonic acid and 1 or 2 drops *only* of 10 per cent ferric chloride to produce a purple colour. The solution must be prepared afresh each time and is stable only for a few minutes.

Add the gastric filtrate drop by drop to a 1 in. column of the reagent in a boiling tube till no further colour change takes place. A canary yellow colour due to ferric lactate indicates the presence of lactic acid. The test should be done by daylight. Distilled water is essential.

Qualitative tests for lactic acid are unsatisfactory. More elaborate methods than Uffelmann's have been devised, but it is debatable *whether they are of practical value clinically*. MacLean has adapted Hopkins' thiophene test for use with gastric contents, and Ege has introduced a method of quantitative estimation of lactic acid, utilising his determination of the relative solubility of lactic acid in water and in ether. If free HCl is found in more than traces tests for lactic acid are superfluous, since lactic acid is not formed if free HCl exceeds 20 c.c. N/10 per cent.

### Bile Pigment (Bilirubin)

Bile may be obvious from the naked eye appearance after a test meal of oatmeal gruel. Toast, however, often gives rise to a brownish colour, apart from bile. If the gastric contents be allowed to stand, a green colour may develop spontaneously, making tests for bilirubin unnecessary. This green colour is due to biliverdin,

which has been formed by the action of the oxygen of the air on bilirubin. The detection of bilirubin depends either on its oxidation to biliverdin and other coloured oxidation products, or on the formation of azobilirubin, which is an indicator of a purplish red hue in moderately acid solution. The bile may be in the filtrate, or adsorbed on the precipitate.

(a) *Examination of Filtrate by Iodine Ring Test* Perform a ring test, floating tincture of iodine (see Appendix) diluted with an equal quantity of distilled water, on the surface of the filtrate. A green ring indicates bile pigment.

(b) *Examination of Precipitate by Gmelin's Test* To some of the precipitate on the filter paper add 1 or 2 drops of concentrated nitric acid. If bile be present, a play of colours will result, ranging from yellow to blue, green or blue being the essential colour.

Nitric acid may be used as a ring test with the filtrate (cf examination of urine for bile pigment, Chapter II).

(c) *Van den Bergh's Test* (cf Chapter XII) (i) Extract a portion of the precipitate with 3 to 5 c.c. of alcohol by shaking well and filtering or centrifuging. (ii) (If the gastric filtrate is alkaline to about 3 c.c. add hydrochloric acid (about N/10) drop by drop till acid to litmus.) To the acid gastric filtrate, about 3 c.c. add 2 volumes of alcohol about 6 c.c., mix, and filter or centrifuge.

To the alcoholic extract from (i) or (ii), add  $\frac{1}{2}$  volume of Van den Bergh's freshly prepared diazo reagent (cf Chapter XII) and allow to stand for a few minutes, till the maximum colour has developed.

A red or purplish red (or purple blue if the original gastric contents were very acid) colour is obtained if bilirubin (or biliverdin) be present.

### Blood

(a) *Benzidine Test* Place a 1 in. column of the gastric fluid in a test tube taking care to include any red or brown specks which look like blood and boil. Cool and neutralise with NaOH (approximately N/10) if acid using litmus paper as external indicator. Perform the benzidine test with some of this suspension as described under occult blood in faeces (Chapter XXIV).

The benzidine reaction may not succeed in suspensions containing free hydrochloric acid, hence the necessity for neutralising. If there is any product of red meat in the gastric contents the test will be positive apart from blood. The test is therefore of no significance if meat fibres be noted in the microscopical examination.

The benzidine test is really too sensitive when applied to gastric contents. Positive results are the rule rather than the exception, and often are probably due to trauma by the stomach tube though the writer has found that the original oatmeal gruel itself gives a weakly positive benzidine reaction (after boiling). In clinical work the naked-eye appearance and microscopical examination usually

suffice, though the nature of any doubtful brown lumps may be decided by the acid porphyrin test

The *pyramidone test* (cf Chapter II) is unsuitable for gastric contents because HCl alone gives a positive result

(b) *Acid Porphyrin Test* In the presence of red or brown specks, or of "coffee grounds," the simplest and most rapid test is the following Separate the specks or "coffee grounds," together with other suspended matter, by centrifuging Add the sediment drop by drop to a  $\frac{1}{2}$  in column of concentrated sulphuric acid in a test tube, and shake to dissolve Note the colour of the solution and examine it spectroscopically If blood is present, acid protoporphyrin is formed, and the solution becomes a reddish purple and shows the characteristic absorption bands (For spectrum see p 212)

(c) *Hæmochromogen Test* Separate any brown or red lumps by centrifuging as in (b) Treat the deposit and a few cubic centimetres of supernatant fluid with 40 per cent NaOH until strongly alkaline Warm, cool and add a few drops of yellow ammonium sulphide<sup>1</sup> Examine with the spectroscope for the absorption spectrum of hæmochromogen (cf p 212)

The brown lumps or specks in, or the brown colour of, gastric contents containing "altered blood" are due to hæmatin, acid neutral or alkaline according to the reaction Commonly the hæmatin is in suspension and is thrown down by centrifuging but sometimes it is in solution or colloidal suspension The above tests are important because a brown colour may be due to the pigment of food or drink, e.g., coffee, tea, cocoa, toast, etc

## Starch

To a few drops of the unfiltered gastric contents add a very dilute solution of iodine (N/50 or even more dilute) drop by drop, and shake A blue colour demonstrates the presence of starch, which may be in solution or enclosed in some of the white lumps in suspension

## Quantitative Estimation of Free HCl and Total Acidity

If the gastric filtrate is alkaline or neutral to litmus, obviously the acidity is *nil* If acid, proceed as follows —

(a) *Using Thymol Blue* To 10 c.c. of filtrate in a boiling tube add 4 or 5 drops of 0.1 per cent thymol blue, and titrate with N/10 sodium hydroxide till the solution is no longer red (free HCl neutralised) The colour sequence is red, orange, yellow The titration is complete when reddish-orange gives place to yellow orange, i.e., when there is no longer any red tinge Suppose  $x$  c.c. of NaOH are required Then continue the titration, when the solution will change from yellow to green and finally to blue

<sup>1</sup> Or a knife point of solid sodium hydrosulphite  $\text{Na}_2\text{S}_2\text{O}_4$  and a drop of ammonia solution in case the gastric sample lacks a N containing substance to combine with alkaline reduced hæmatin to yield a hæmochromogen



Titrate just to a full blue, when the total acidity will have been neutralised. Suppose a total of  $y$  c.c. of NaOH are required.

**Calculation** Since 10 c.c. of filtrate are used, 100 c.c. of gastric juice will contain  $10 \times x$  c.c. of N/10 free HCl, and  $10 \times y$  c.c. of N/10 total acid or  $10 \times x \times 0.00365$  gm. of free HCl per cent, and  $10 \times y \times 0.00365$  gm. of total acid per cent calculated as HCl. (For ready reckoner, converting c.c. N/10 per cent into gm. per cent (as HCl) see Appendix.)

If 10 c.c. of filtrate cannot be obtained, or is not available as in the fractional method, the titration may be performed on 2 c.c. of filtrate using a 2 c.c. microburette for the caustic soda. Alternatively an ordinary burette and 0.01 N alkali may be employed.

Cole and Adie introduced a more accurate, though more elaborate method of performing the above titration, using standard solutions of hydrochloric acid *plus* thymol blue for comparison. In clinical work however this is really unnecessary, and is often not very much more accurate owing to the difficulty of matching with standards gastric filtrates which are either not quite clear or tinged with bile.

Thymol blue is a double range indicator and covers both free HCl and total acidity. Alternatively, Gunzburg's reagent may be used as an external indicator for free HCl followed by phenolphthalein for total acidity. The method is however, too time consuming for routine use. Similarly Topfer's reagent may be used for free HCl followed by phenolphthalein for total acidity. The method is not quite so good as the thymol blue method, but has been widely employed.

(b) *Using Topfer's Reagent and Phenolphthalein* To 10 c.c. of filtrate add 4 or 5 drops of Topfer's reagent, and titrate with N/10 NaOH till all the red colour has gone—free HCl neutralised by  $x$  c.c. of NaOH. The colour sequence is the same as with thymol blue *viz.*, red, orange, yellow and the end point is the same, *i.e.*, when there is no longer any red tinge. Then add 3 or 4 drops of 1 per cent alcoholic phenolphthalein and continue the titration till a definite red tinge replaces the yellow—total acidity neutralised by  $y$  c.c. of NaOH. The calculation is the same as that given under the thymol blue method. The difficulty with both the methods described is that the end points with gastric juice are frequently not sharp. This is perhaps even more true for the second than for the first method. The second method tends to give slightly higher results as will be understood from the respective pH ranges of the indicators —

Thymol blue	1.2 to 2.8 and 8.0 to 9.6
Topfer's reagent	2.9 to 4.2
Phenolphthalein	. 8.3 to 10.0

When recording the figures, the results should be expressed as so many c.c. of N/10 acid per 100 c.c. of gastric contents, or so many gm. per cent (as HCl). It is inaccurate to express the results

as so many c c of N/10 NaOH per cent, because gastric juice does not contain NaOH, though this last notation will frequently be found in text books and in medical literature!

Whenever there is any doubt as to the presence of free HCl, always perform Gûnzburg's reaction (p 437) and decide from the result obtained by that test

### Total Chlorides

To 10 c c of filtrate add 15 c c of N/10 silver nitrate about 2 c c of concentrated nitric acid, and 5 or 6 drops of saturated potassium permanganate. Boil. Add more permanganate drop by drop, boiling between each addition till the fluid is well decolorised. Avoid adding an excess of permanganate, which will produce a permanent pink. (If this should happen a trace of solid potassium oxalate may be added to remove the excess of permanganate, but with a little practice this should rarely be necessary). Cool thoroughly. Add 4 or 5 drops of 30 per cent ammonium iron alum solution, and back titrate with N/10 potassium thiocyanate solution.

Example of calculation. To 10 c c of filtrate 15 c c of N/10  $\text{AgNO}_3$  were added. Back titration with N/10 potassium thiocyanate, 4.7 c c

1 c c of N/10 KCNS reacts with 1 c c of N/10  $\text{AgNO}_3$ ,

4.7 " " " " 4.7 " " "  
 $\text{AgNO}_3$  combined with chlorides equals  $15 - 4.7 = 10.3$  c c N/10  
 10 c c of gastric filtrate contain the equivalent of 10.3 c c of  
 N/10 chloride,

100 " " " " " equivalent of 103 c c of  
 N/10 chloride

Result. Total chlorides equal 103 c c N/10 per cent or 0.376 gm per cent calculated as HCl

### Mineral Chlorides.

Place 10 c c of gastric filtrate in a platinum dish and heat to dryness on a boiling water bath or electric hot plate. Heat carefully with a Bunsen till the contents are completely carbonised, then heat well till a grey black ash results, but do not raise to red heat, lest inorganic chlorides be volatilised. Wash the ash quantitatively into a small beaker with distilled water, and add 10 c c of N/10 silver nitrate, about 2 c c of concentrated nitric acid, and 5 or 6 drops of 30 per cent ammonium iron alum. Mix well, stand for a few minutes and back titrate with N/10 potassium thiocyanate. The calculation is similar to that given under total chlorides.

Example. Back titration was 4.6 c c. Therefore mineral chlorides in 10 c c of filtrate =  $10 - 4.6 = 5.4$  c c N/10. Result. Mineral chlorides equal 54 c c N/10 per cent, or 0.197 gm per cent calculated as HCl.

Active acid (HCl) equals total chlorides less mineral chlorides. Therefore active HCl equals  $103 - 54 = 49$  c c N/10 per cent or 170.9 gm per cent HCl.

In estimating mineral chlorides the filtrate is ashed so that free HCl and HCl combined with proteins is volatilised (*cf* also footnote on p. 435)

The above methods may be used for 5 c.c., 2 c.c., or even 1 c.c. of gastric filtrate with proportionately smaller amounts of reagents, using a microburette for the thiocyanate. Patterson has modified the technique so that 0.2 c.c. of gastric filtrate is employed in each of the two determinations. This modification is recommended for fractional analysis.

#### Total Chlorides (Patterson's micro-technique)

Measure accurately 0.2 c.c. of gastric filtrate, using a blood pipette calibrated "to contain". Discharge into 3 c.c. of pure nitric acid in a wide test tube, washing out the pipette by sucking up the acid three or four times. Add 1 c.c. of N/30 silver nitrate, 4 c.c. of pure acetone and 3 drops of saturated iron alum solution. Mix well and back titrate with N/30 ammonium thiocyanate in 67 per cent alcohol.

The acetone is added to make the end point sharper. The N/30 silver nitrate is conveniently prepared from an N/10 solution by dilution with water and the thiocyanate from an N/10 solution by dilution with about 2 volumes of absolute alcohol. The thiocyanate must be checked against the N/30 silver nitrate in the usual way.

**Calculation.** Using solutions of exactly N/30 concentration, if  $x$  be the number of c.c. required in the back titration, then  $(1 - x) \times \frac{100}{0.2} \times \frac{1}{3}$  equals the number of c.c. of N/10 chloride per 100 c.c. of gastric filtrate. To convert into gm. per cent as HCl, multiply by 0.00365 (see Appendix).

#### Mineral Chlorides (Patterson's micro-technique)

Measure 0.2 c.c. of gastric filtrate into a small thin walled porcelain crucible with the aid of a blood pipette. Blow out the contents of the pipette as completely as possible, and then rinse out with 3 or 4 drops of distilled water, adding these washings to the crucible. Evaporate to dryness on a boiling water bath or electric hot plate. Heat the crucible carefully over a Bunsen till the contents are completely carbonised, continue heating for another five minutes, but do not raise to a dull red heat, lest mineral chloride be lost by volatilisation. Cool and wash out the grey black ash into a wide test tube with 2 or 3 c.c. of distilled water. Add an equal volume of nitric acid, 1 c.c. of N/30 silver nitrate, 4 c.c. of acetone, and 3 drops of saturated iron alum solution. Shake well and back titrate with N/30 alcoholic thiocyanate.

The calculation is the same as for total chlorides. The only part of the procedure that requires much practice is the ashing. If the temperature is not raised sufficiently high there is a risk of protein HCl not being driven off. If a dull red heat is attained, mineral chloride is often volatilised.

Pepsin is estimated by digesting either egg white (Mett's tubes) or hemp seed protein (edestin), or the globin of a hæmoglobin solution (Anson and Mirsky) For the details of technique the reader is referred to larger works

### Microscopical Examination.

This is hardly a chemical procedure, but the chemical pathologist often must be able to carry it out in order to interpret fully some of his chemical findings, e.g., when tests for blood are positive

A drop of the deposit, obtained by centrifuging or by allowing it to settle to the bottom of the container, is mounted under a cover slip, and examined for —

- (a) Cells (W b c, R b c, epithelial cells and tumour cells)
- (b) Food residues (starch granules, meat fibres, vegetable remains, charcoal granules, casein curds, etc.)
- (c) Organisms (Boas Oppler bacilli, sarcine and yeasts)

As previously mentioned, the fasting juice is really the only sample worth examining microscopically After a meal, little is seen as a rule except the remains of that meal After an Ewald meal the field is usually a mass of starch granules, together with a lesser number of yeast cells, both derived from the toast In infants, after milk feeds, casein curds alone are visible Of course, in all samples there are numerous oily droplets of the liquid paraffin used for lubricating the tube

Leucocytes (w b c) are almost a constant finding in the resting juice, due in most cases to the swallowing of saliva Certainly groups of leucocytes in strings of mucus may safely be discounted, but occasionally there is true pus, clumps of leucocytes in varying stages of degeneration and not surrounded by mucus This is a common finding in carcinoma of the stomach

The criteria laid down (see "Urinary Deposits," Chapter IV) for the identification of erythrocytes (r b c) must be carefully observed otherwise globules of liquid paraffin and amorphous debris may mislead the beginner The significance of red blood corpuscles has already been discussed under the heading of blood On the whole, corpuscles are probably not so significant as altered blood Swallowed corpuscles, however, will rapidly be lysed, and acid hæmatin " (altered blood) " will be formed if there is more than a trace of free HCl in the stomach For this reason one cannot expect to see erythrocytes when the samples received for analysis are strongly acid

As mentioned before, squamous epithelial cells are of no significance, being derived from the mouth or pharynx, but gastric epithelial cells are pathological Occasionally large endothelial cells which contain numbers of phagocytosed black particles are seen in gastric deposits These cells may also be seen in sputum, and are derived from the lungs, the black particles being pieces of carbon inhaled with dust This point is of importance, because such cells might wrongly be interpreted as wandering cells with phagocytosed blood pigment originating from the stomach Fragments of gastric

epithelium or isolated gastric epithelial cells may be seen in carcinoma ventriculi or in gastritis. Tumour cells are very rarely seen, but are diagnostic.

Starch granules are readily recognised by their concentric lamination, or, if necessary, by adding iodine which turns them blue black. As previously mentioned, starch is always found one hour after an Ewald meal. Meat fibres are likewise easily detected by their transverse striation and brown colour (*cf* Fig on p. 272). The walls of the cells in vegetable remains are thick and refractile (Chapter XXIV). Casein curds appear as masses of amorphous white fluffy particles of different sizes, once seen they are easily recognised. In the fasting juice none of these food residues is normally present, except for occasional vegetable remains. In carcinoma and pyloric obstruction residues of food may be found which, from the history, must have failed to pass the pylorus for many hours, or even several days.

Normally when a charcoal biscuit is given the previous night, no trace of charcoal granules should be found in the fasting juice next morning. The finding of charcoal, therefore, has the same significance as the discovery of food residues. In the fasting juice food residues are looked for in order to obtain some indication of the motility of the stomach or of the functioning of the pylorus.

The discovery of Boas' Oppler bacilli was at one time thought to be an important point in the diagnosis of carcinoma, but it is now generally agreed that the examination of the flora of the stomach in any gastric sample is of little or no value clinically. Sarcinae are sometimes observed in simple dilatation of the stomach and other conditions, but are generally absent in carcinoma, and it was this last point that used to be considered of value. As mentioned before, yeast cells are commonly seen in the one hour test sample and are derived from the toast, they are of no significance.

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## CHAPTER XXIII

### THE CHEMICAL EXAMINATION OF THE DUODENAL CONTENTS

Books and General References. Lyon's *Non surgical Drainage of the Gall Tract*

Chiray and Lebon's *Le Tubage Duodénal*

Einhorn's *The Duodenal Tube*

Meakins, J, *Brit Med J*, 1922, 1, 983

Kellogg's *The Duodenum*

DUODENAL intubation may be employed in order to study the secretions of the duodenum, of the pancreas (cf Chapter XIII), or of the liver (cf Chapter XII). Unfortunately, mixtures of these fluids in varying proportions, together with unknown amounts of gastric contents which have passed through the pylorus, are obtained. Some degree of separation may be secured by first emptying the stomach; secondly, aspirating the duodenum, and, thirdly, stimulating the output of bile by administering magnesium sulphate solution and then aspirating again. Lyon has claimed that it is possible to obtain fractions after the magnesium sulphate which correspond to the bile, A from the bile ducts, B from the gall bladder, and C from the liver, but other workers do not agree that the bile from these three sources can be fractionated successfully. The duodenal tube may also be employed therapeutically, either as a means of draining the bile passages, or to introduce food or drugs directly into the duodenum, or for washing out the duodenum.

The fluids aspirated may be examined bacteriologically (e.g., for the typhoid bacillus and other organisms), microscopically, or chemically. Under the chemical examination may be included tests for certain drugs, a line of investigation which has been little explored, and which may provide information of great pharmacological and physiological interest and of clinical value. The excretion in the bile of urotropin, of phenoltetrachlorophthalein and other dyes (cf Chapter XII), and of arsenic after injection of organic arsenic compounds (Martens and Koers) may be cited as examples.

The chief value in clinical work of chemical examination of the duodenal contents is in diseases of the pancreas and its ducts, or of the bile ducts and gall bladder.

#### THE TECHNIQUE OF INTUBATION

The duodenal tube is similar to a stomach tube, but longer. It is usually of the flexible type with a metal olive at one end (Einhorn, Rehfuess or Ryle's pattern), and a syringe, or apparatus for continuous suction, at the other end. It is marked with rings,

as in the case of the stomach tube (see Chapter XXII), and also at distances of 28 and 32 in from the olive. The length of tube from the duodenum to the teeth varies, but is commonly about 28 in.

After a night's fast, the tube is passed into the stomach (for instructions, see Chapter XXII), and the gastric residuum is removed as completely as possible. The stomach is then washed out with warm water, the washings being aspirated thoroughly. The patient is then instructed to lie on his right side, and to read a book or otherwise to divert his attention pleasantly. It is important for the operator to employ all means in his power to counter the patient's nervousness. Time is then allowed for the natural movements of the stomach to force the metal olive and the end of the tube into the duodenum. Usually this takes from half to two hours. The passage into the duodenum is best verified radiologically, but may frequently be ascertained by withdrawing a little fluid from time to time with the syringe. When bile stained fluid which is alkaline in reaction is obtained the tube is probably in the duodenum. To check this the patient may be given a little coloured water, milk, or wine to drink. If this can be recovered immediately by aspiration, the tube is still in the stomach.

The duodenal contents are aspirated as thoroughly as possible. Lyon recommends the preliminary injection of about 30 cc of air down the tube to prevent the mucous membrane becoming sucked tightly against the olive.

In order to recover bile from the bile passages, 50 to 100 cc of 25 per cent magnesium sulphate are injected down the tube. Lyon finds that magnesium sulphate is the best stimulant, and then in order olive oil (1 oz) and 10 per cent peptone, though many other substances (sodium sulphate, sodium citrate, sodium chloride, calomel, glucose solutions, hydrochloric acid, etc) apparently act similarly to a lesser degree. Bile may be secured in from two to ten minutes. Lyon's description of the three fractions is as follows. The colour of the first fraction is pale yellow, its volume is normally about 10 to 30 cc, and the time required for its extraction is about one to three minutes from the start of the flow. As soon as the colour deepens noticeably the collection of the second sample is begun. Normally, 10 to 75 cc can be obtained. As aspiration progresses the bile flows more intermittently, and becomes darker and more viscid. When the fluid again becomes lighter in colour the collection of the third sample is started. The bile is now obtained very intermittently, and is thinner and usually transparent. From a few cubic centimetres to several ounces may be obtained.

The whole process frequently occupies three or more hours, and this is the main objection to its clinical employment. Occasionally, owing to spasm or obstruction of the pylorus, it is impossible to enter the duodenum. It is then advisable to verify the position of the tube radiologically. Pyloric spasm may be overcome by an injection of atropine, or by a course of belladonna for the three or four preceding days. Simple kneading of the stomach from left to



right may produce the desired result. The olive generally lodges finally in the second part of the duodenum, but occasionally may reach even further if too great a length of tube is swallowed.

In addition to an examination of the fasting duodenal contents, fractional tests may be carried out after a meal, on the same principle as fractional analysis of the stomach contents (Baird *et al*).

If little or no fluid is obtained, stimulation by water (1 oz.), dilute alcohol (10 to 30 c.c. of 10 per cent.), N/10 hydrochloric acid (about 5 c.c.) or ether (1 to 4 c.c. injected slowly down the tube) may be tried. The subcutaneous injection of secretin has also been recommended. Usually these provocative measures are unnecessary, but should be employed before concluding that the absence of secretion is due to a complete obstruction of the pancreatic and of the bile passages. Ether causes congestion of the mucosa, so it might start a serious hæmorrhage if a duodenal ulcer were present.

### EXAMINATION OF SAMPLES WITHDRAWN

The examination of the gastric residuum has already been described in the last chapter. The stomach washings may be discarded. The investigation of the duodenal contents obtained without stimulation, and of the bile obtained after magnesium sulphate, requires special description.

In each case the reaction to litmus, the volume obtained, the colour, and the naked eye appearance are carefully noted. Whether the fluid be transparent, or opalescent, or contains flakes or lumps of mucus or clots of blood, or "calculi," is observed, and any peculiar particles are removed for microscopical examination. In addition each sample is centrifuged, and the sediment (if any) is inspected under the microscope. This should be done within a few minutes of the recovery of each sample, since the cells may be digested rapidly by the enzymes. It may be said at once that this preliminary macroscopic and microscopic investigation often gives as much or more information than the more elaborate chemical tests.

#### The Duodenal Juice (no magnesium sulphate)

The *alkalinity* may be measured by titration of a known volume of duodenal fluid with N/10 hydrochloric acid. Such a wide range of variation is obtained, however, that the estimation is of little value. This is to be expected owing to the difficulty of preventing ingress of gastric juice. Damade, using methyl orange as indicator, obtained values ranging from 14 to 125 c.c. of N/10 alkali per 100 c.c. of duodenal juice. Qualitative tests with indicators (litmus, phenolphthalein, bromthymol blue, phenol red, or methyl orange, etc.) are useful as a rough guide to the reaction (for pH ranges of these indicators see Appendix), in that they give information as to the likelihood of admixture with gastric juice, and as to the necessity for adjusting the reaction before testing for enzymes, etc.

For clinical purposes the colour is generally a sufficient indication of the presence and approximate quantity of bilirubin. Qualitative chemical tests may be performed as for bilirubin in urine (see Chapter II), and quantitative methods as for bilirubin in blood (see Chapter XII, and Chiray and Lebon's book).

For bile-salts the same tests may be used as with urine (see Chapters II and XII), or a stalagmometer may be employed to measure the number of drops given by 1 c.c. of duodenal fluid, the apparatus having been previously calibrated with distilled water. Carnot and Mauban have applied Hay's test to a series of dilutions of duodenal juice, and have thereby obtained an approximately quantitative measurement (see Chiray and Lebon's book).

For methods of detecting blood, see Chapter XXII.

Cholesterol may be estimated by the technique used for blood cholesterol (Chapter XIX).

An increase of proteins may be shown by the usual tests (see Chapter II), and occurs in infective or suppurative lesions of the duodenum, gall bladder, biliary or pancreatic ducts.

Tests for enzymes, however, are the most important of all the chemical methods of examining duodenal juice, in that they give the most information about the external pancreatic secretion. Examination should be made on the day of the intubation. Coope and others maintain that qualitative tests alone are worth making owing to the unknown dilution of pancreatic juice by bile, by duodenal secretion, gastric juice, etc. Of the qualitative tests it is generally agreed that those for diastase are of the least value, owing to the risk of admixture with salivary ptyalin. For trypsin the X-ray plate method given in the next chapter and for lipase the method of Carnot and Mauban (see Chapter XIII), may be recommended as simple. Systems of quantitative analyses for all three enzymes have been devised by Lueders and Bergeim (see Chapter XV of Lyon's book), and by Okada *et al.*, but since further investigation is required before their true clinical value can be assessed, the reader is referred for details to the papers quoted and to Chiray and Lebon's book. A point not mentioned by any of the above authorities is that diastase may readily be estimated by Wohlgemuth's method (see Chapter XIII).

#### Bile obtained after Stimulation by Magnesium Sulphate

The same chemical tests may be applied, though, in general, examination for bile pigments and bile salts are important, whilst tests for enzymes are of no value. The most important data are obtained from the volume and general appearance, and by the microscopical and bacteriological examinations. The last is outside the scope of this book, and the macroscopical examination has already been discussed. There remains for description, therefore, the microscopical examination. Lyon claims that separate examinations of the A, B, and C bile often show in which part of the gall tract the pathological lesion exists.

## THE MICROSCOPICAL EXAMINATION OF DUODENAL FLUID OR BILE

The centrifuged deposit of a normal fasting duodenal juice contains more epithelial cells than anything else. Some of these are squamous (from the mouth, etc.) and are of no significance. The rest are cylindrical or irregular in shape, and are derived from the stomach and duodenum, or rarely from the biliary or pancreatic passages. There are often a few leucocytes and a fair quantity of mucus. According to some workers, it is not uncommon to find a very few crystals of cholesterol, calcium bilirubinate, or calcium oxalate in health (*cf* Hunt). There are no food residues.

In catarrhal jaundice there is an increase mainly of epithelial cells. In infective and suppurative conditions of the bile passages, pancreas or duodenum there is a general increase of cells, with a large number of leucocytes and occasionally red blood corpuscles. In cholelithiasis there is sometimes an excess of cholesterol crystals and it is to secure this observation in particular that duodenal intubation is undertaken when the presence of gall stones is suspected clinically. In certain cases of cholelithiasis "calculi," or minute calculi may be visible to the naked eye as little brownish granules entangled in mucus. These are fairly readily compressed between cover slip and slide and may or may not show the typical notched crystals of cholesterol (Fig 19, p 57) when viewed under the microscope. They are partially soluble in ether and chloroform, and it is possible that, when no crystals are observed, the soluble matter is mainly amorphous cholesterol.

## THE VALUE OF THE TESTS IN CLINICAL WORK

As already mentioned, as a general rule the observations made whilst passing the tube, the naked eye appearance of the samples withdrawn and the microscopical examination (combined with bacteriological examination, where indicated) provide the most important evidence of abnormalities, and particularly of inflammatory or suppurative lesions. The chemical examination is not so useful partly because similar analyses of other fluids (*e.g.*, blood or urine) often produce the desired evidence more simply, and partly because the interpretation of results is difficult. Thus in pancreatic disease, examination for ferments is the most important, but whilst there is generally no difficulty in assessing the meaning of their complete absence or gross diminution, it is often difficult to know what significance to attach to the finding of small or moderate amounts. Quantitative methods are not really satisfactory, and for this reason it is impossible to obtain the range of variations in health as a basis for comparison with the findings in disease. None the less, the methods are of some value as an extension of the clinical examination as indicated in the following examples.

When clinically obstruction of the pancreatic duct (by malignant growth, stone, etc.) is suspected, the demonstration that the duodenal fluid contains no trypsin and no lipase is important confirmatory evidence, provided that the duodenal intubation, etc., is successful. Again, when clinically there is a swelling in the region of the head of the pancreas, the absence of the ferments from the duodenal juice localises the tumour. The inability to obtain bile stained duodenal juice from a jaundiced patient is an indication that the obstruction to the bile passages is complete, but a similar conclusion would probably have already been reached from an examination of the feces. The investigation of the duodenal contents, however, might reveal cholesterol crystals or "calculi," thus indicating that the absence of bile is due to the impaction of a gall stone somewhere in the common bile duct. Conversely, the same discovery in duodenal contents which are bile stained points to the presence of gall-stones, either in the gall bladder or elsewhere in the bile passages, but without completely blocking the common duct. The complete absence of both bilirubin and of ferments would suggest an obstruction at the ampulla of Vater, provided the technique can be assured.

The above examples suffice to indicate the value of the method as an extension of the clinical examination. On the other hand, it would be easy to quote examples in which the tests would add no material information. In suspected chronic pancreatitis, for instance, the presence of ferments in the duodenal contents by no means negatives the clinical diagnosis, and the results of attempts to make quantitative estimations are unsatisfactory for the reasons given above. Okada *et al* claim that if continuous evacuation of the duodenal juice is maintained for three hours, the analyses do reveal significant variations from the "normal" if the volume of juice is taken into consideration, but their work needs confirmation, and, owing to its great length, their method is unlikely to receive widespread clinical trial.

Lastly, some of the published results of analyses will give further indications of the clinical possibilities of duodenal intubation in diagnosis.

**Bilirubin** (Churay and Lebon) Little or *nil* in obstructive jaundice. Increased in familial jaundice, chronic splenomegalic icterus, hæmolytic jaundice, paroxysmal hæmoglobinuria and pernicious anæmia. In catarrhal jaundice the concentration of bilirubin may be normal, but the total output low.

**Bile-salts** There may be an increase or decrease in parallel with bilirubin, or there may be "dissociation," as in hæmolytic jaundice.

**Urobilin and Urobilinogen** (Churay and Lebon) Low in anæmia due to hæmorrhage, in cancer, in tuberculosis and in biliary lithiasis. High in hæmolytic anæmia and in pernicious anæmia.

**Cholesterol** (Churay and Lebon) In health 50 to 60 mgm., in biliary lithiasis 70 and 71 mgm., and in pernicious anæmia 107 and 152 mgm. per 100 c.c., are quoted.

Fox quotes Pribram's figures for liver bile (C bile) in thirty-seven experiments, as follows —

Total solids, 1.2 to 2.9, average 1.75 per cent

Cholesterol, 30 to 150, average 50 mgm per 100 c c

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## CHAPTER XXIV

### THE CHEMICAL EXAMINATION OF THE FÆCES

Books *Cambridge's The Fæces of Children and Adults*

Chapter on Fæces in Hawk and Bergeim's *Practical Physiological Chemistry*

Langeron and Rondeau du Noyer's *Coprologie Microscopique*

Schmidt and Strasburger's *Die Fæces des Menschen*.

SEVERAL examples of the value of chemical examination of the fæces have already been given in preceding chapters, particularly in reference to efficiency tests of the liver (Chapter XII) and of the pancreas (Chapter XIII). It is now necessary to give details of the technique of the simpler methods, and the interpretation of the results in other diseases. For convenience in reference the chemical constituents will be considered alphabetically, after the methods of collection and of preservation, and the macroscopical and microscopical examination, have been discussed.

#### COLLECTION OF FÆCES

The usual method of collection in a bedpan needs no special description. In laboratory work it is often convenient to place in the lavatory pan an evaporating basin of 9 in. or 10 in. diameter, thus saving a transference of the fæces. For most chemical tests it is best to send the complete specimen to the laboratory, and this is particularly necessary when fat analysis has to be made. Care should be taken to avoid contamination by urine, and enema results are of no value. The specimen should reach the laboratory within an hour when tests of its reaction or for trypsin are to be made. Otherwise, intervals of a few hours may be allowed.

In metabolism experiments, and in other circumstances in which the fæces corresponding to a particular diet or method of treatment are to be collected, "markers" are employed. These usually consist of powdered charcoal (0.2 to 0.5 gm.) or carmine (0.2 to 0.5 gm.). The marker is given in a cachet or gelatin capsule at the beginning and at the end of the period to be studied. The fæces are coloured grey by the charcoal and red by the carmine. Other substances, such as Sudan III, have been employed. The sample coloured by the first, but not the portion coloured by the second marker, is included in the complete collection.

#### PRESERVATION OF FÆCES

As a general rule fresh specimens should be examined. When delay is unavoidable, alterations may be minimised by storage in

the ice chest. In certain experiments the faeces may be covered with absolute alcohol (*e.g.*, prior to analysis for fats). Often it is best to dry each sample on the water bath soon after it is obtained, and then to powder and combine all the dried portions which may be stored for future analysis (*e.g.*, in fat and mineral metabolism experiments). Cammidge recommends formalin as a preservative when the faeces have to be sent long distances. Whenever a preservative is added the analyst should be informed of its nature.

### GENERAL COMPOSITION AND MACROSCOPICAL EXAMINATION

Following Coope (*Lancet*, 1921, ii, 9) the normal stool of an adult on an ordinary mixed diet may be defined as a moulded, fairly firm, more or less cylindrical body, reaction neutral or slightly alkaline to litmus, coloured brown chiefly by stercobilin and containing 70 to 80 per cent. of water, large quantities of bacteria, and a small proportion of food residues, mainly cellulose remains and partially digested muscle fibres. In more detail, normal faeces consists of water bacteria, many of which are dead, the remains of the secretions of the digestive tract, substances excreted into the bowel (such as salts of calcium, of iron, etc.), small amounts of food residues which cannot be digested or are incapable of or have escaped absorption, and cellular detritus resulting from the normal death and shedding of the lining epithelium.

In disease the faeces may contain things they ought not to contain—notable quantities of food residues, abnormal substances derived from the gut walls, such as blood, serum, pus, mucus, or from the lumen such as intestinal calculi, “sand,” or parasites—or they may not contain the things they ought to contain, particularly stercobilin.

The form, consistency (liquid, semi liquid, semi solid, solid, scybalous), colour, odour and general characteristics are observed, and any peculiar masses—shreds, flakes, suspected parasites, etc., are withdrawn for microscopical or chemical examination.

Further information may be obtained by emulsifying a portion, about 1 in. in diameter, in warm physiological saline. Samples of this emulsion are placed in a Petri dish, and further diluted with the saline if necessary, and examined against a background partly white and partly black. “Various things may be seen—vegetable debris, tiny brown rods of muscle fibre, actual masses of undigested meat, white dots of the so called ‘potato granules,’ parasites, possibly epithelial debris, more notably, typical white fragments of connective tissue and flakes of mucus.” Suspected fragments are removed for special examination.

The faeces of infants on a milk diet are soft, semi liquid and of a bright canary yellow colour, but those of older children have the same general appearance as the adult stool. The colour of stools is discussed more fully later.

## MICROSCOPICAL EXAMINATION

In routine work all that is necessary is to emulsify a speck of faeces in a drop or two of 0.65 per cent NaCl on a slide, cover with a slip, and examine under a one sixth objective. With a little experience it is easy to avoid making the suspension either too thick or too thin. Several preparations should be made and examined, and any suspicious looking particles should be selected for identification.

To avoid shrinking of red blood corpuscles 0.65 per cent NaCl is preferred to physiological saline, 100 gm of fresh faeces contain some 2 to 3 gm of salts, since about 1 part of the faeces in 20 parts of saline makes a suitable suspension for microscopical examination, if 0.85 per cent NaCl is used the mixture is hypertonic making red cells more difficult to identify.

In certain circumstances it is useful to make a second suspension in Lugol's iodine, which turns starch (granules) blue, and stains cells, muscle fibres, etc., brown.

A list of the more important items for which a search may be made are given below.

*Microscopical Examination of Faeces*

Food residues	{	Meat fibres { undigested
		(Fig on { partially digested
		p 272 ) { almost completely digested
		Fat globules (beware of liquid paraffin and other oils)
		Fatty acid crystals (acicular)
		Soap plaques and crystals
Cells	{	Starch granules
		Cellulose (fruit and vegetable) residues
		Red blood corpuscles
		Leucocytes
Crystals	{	Epithelial cells
		Triple phosphate (ammonium magnesium)
		Calcium oxalate
		Cholesterol and coprosterol
Ova	{	Charcot Leyden crystals
		Oxyuris vermicularis
		Ascaris lumbricoides
		Trichocephalus dispar
		Ankylostoma duodenale
		Bilharzia (lateral spine)
Parasites or cysts	{	Etc
		Derived from or parents of ova listed above
		Entamoeba histolytica
		Lambha intestinalis
		Etc
Mucus and foreign bodies (hairs, wool, tow, etc )		
Bacteria		

A description of parasites, their cysts and ova, of foreign bodies, and of bacteria, is outside the scope of this book. The identification of cells has already been given in Chapter IV. The faecal suspension is made in 0.65 per cent NaCl to avoid lysis of erythrocytes. Identification of cells is often more difficult in faeces than in urine, owing to the large quantity of bacteria and of amorphous matter,



and the presence of food residues. Special attention should, therefore, be given to the points made in Chapter IV.

The identification and significance of muscle-fibres (see Chapter XIII and Fig. on p. 272) and of starch granules (Chapters IV and XIII) have already been described.

Fat globules (see Fig. 76) tend to rise to the surface of the preparation. They vary in size, are highly refractile and look "oily." Before deciding that oily globules consist of neutral fats (stearin, palmitin, or olein), it is important to make sure that the patient has not received liquid paraffin, castor oil, or other oily drugs for two or three days previously.

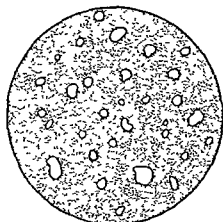


FIG. 76. Globules of neutral fat (fæces).

Fatty-acid crystals (see Fig. 77) (palmitic, stearic) are acicular, soluble in alcohol and ether, and are not stained by Sudan III. They are longer than bacilli and often slightly curved. They are frequently collected in groups of two, three or more. They are unaffected by immersion in aqueous solutions of copper salts for a few minutes, because they are insoluble in water.



Soap plaques

Fatty acid acicular crystals

FIG. 77. Soap plaques and fatty acid crystals (fæces).

Soaps (see Figs. 77 and 78, colour plate) appear as plaques with rolled-over edges, or as masses of acicular crystals. They are insoluble in ether. Calcium soaps are insoluble also in water and in alcohol. A simple method of identification consists of making a suspension of a speck of fæces in a drop or two of saturated copper nitrate solution on a slide, covering with a slip, and examining after a few minutes. The soaps are stained green (Fig. 78, colour plate), owing to their conversion into copper soaps. Fatty acid crystals

*Soap plaque partially obscured by amorphous matter*      *Partially digested muscle fibre*

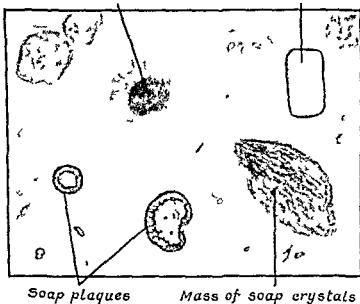


FIG 78 Faecal suspension in copper nitrate solution.  
Soaps stained green

are not stained. Sometimes the fæces decolorise the copper solution, in which case the addition of another drop of the saturated solution or of a crystal of copper nitrate will often succeed.

In health an occasional soap plaque may be visible, but no fat globules and no fatty acid crystals. The presence of numerous soaps and of any neutral fat or fatty acids is pathological, but their significance will be discussed later under the chemical analysis of fæces for "fat."

Cellulose (vegetable and fruit) residues vary enormously in size, shape and appearance, depending on their source. The only way to become really familiar with their origin is to restrict the intake of fruit and vegetables to one particular item for three or four days, and to examine microscopically the corresponding fæces. In general, however, cellulose remains are easily differentiated by their coarse refractile walls and their peculiar shapes (spirals, networks, etc.). Those interested are referred to larger works.

Triple phosphate and calcium oxalate crystals are readily recognised (*cf.* Chapter IV) and are of no special significance. The former may be found in uncontaminated fæces, particularly when alkaline samples have been allowed to stand for some time. Sometimes they are derived from urine which has been allowed to mix with the stool.

Cholesterol crystals (Fig 19, p 57) are not a common finding, and no special significance can be attached to their discovery.

Charcot-Leyden crystals (Fig 79) of the typical diamond form (colourless, elongated octahedra)—similar to those seen in asthmatic sputa—are to be observed in a number of pathological conditions, particularly in amoebiasis, helminthiasis, and mucous colitis, but beyond their common association with these diseases their significance is unknown. Another type of "Charcot Leyden" crystal sometimes seen in pathological fæces is cigar shaped, very similar to typical Charcot Leyden crystals, but without any definite

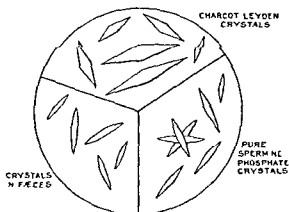


FIG 79 Charcot Leyden and spermium phosphate crystals

angles at the middle (Fig. 79). Morphologically this second type of crystal looks exactly like the typical crystal of spermium

phosphate (Fig 79 on p 457) It is found occasionally in the fæces of cases of enteritis (and of other conditions possibly) at all ages, including infancy There is as yet no proof that either of the two types of crystals in fæces is really spermine phosphate

As a matter of interest it may be noted here that Wrede *et al* (*Zeit f physiol Chem*, 1927, 165, 155) have claimed that the Charcot Leyden crystals in myelogenous leukæmic spleens are spermine phosphate, but in the writer's opinion they have not proved their contention, because they did not separate the crystals from the spleens and show that those crystals were composed of spermine phosphate What they did show was that the leukæmic spleens contained a higher percentage of spermine than the normal spleens they examined, and that the former, but not the latter, contained the crystals

Mixed leukæmic spleens containing crystals may be treated as follows Suspend in 2 volumes of water and centrifuge until most of the crystals are deposited Suspend the deposit in very dilute ammonia solution (1 c c of ammonia S G 0.88 in water to 1 000 c c) and centrifuge again Repeat this washing process several times to separate the crystals as much as possible Store the combined deposits under acetone This treatment must be completed in one day, if the crystals are in contact with water or dilute ammonia overnight they dissolve partially or completely

Further separation is accomplished by repeated elutriation with acetone In this way although there is some loss pure crystals may be obtained

Preliminary observations on such a preparation satisfied the writer that Charcot Leyden crystals are not spermine phosphate nor a phosphate of calcium or of ammonium magnesium nor uric acid The crystals become much more resistant to reagents after storage in acetone They are mainly, if not entirely, organic, and may be a protein

## AMOUNT

The quantity of fæces varies from day to day, and even if observations are extended over several days and the average weight excreted per diem is calculated, the information is of little value clinically since the output varies greatly with the diet, the proportion of water present (diarrhœa, constipation), the amount of bacteria, and so on The following data are taken mainly from Cammidge's book. —

Normal adult on mixed diet	60 to 250 gm of moist fæces in the twenty four hours (Usually 100 to 200 gm )
Pathologically	500 to 1,200 gm
Fasting adult	9.5 gm (Breithaupt), 22 gm (Cetti)
Vegetable diet	370 gm (Rumpf and Schumm)
Flesh diet	Average of 64 gm (Rubner), of 54.5 gm (Harley and Goodbody)
Infants	4 to 120 gm
Young children	60 to 150 gm

Gross bulk (*cf* Chapter XIII) can readily be detected without weighing, and such observations suffice for most purposes in clinical work. Normally about one third of the dried fæces is made up of bacteria.

### BILIRUBIN

Meconium contains biliverdin, and the stools of very young infants generally contain unaltered bilirubin. With the development of the bacterial flora the bilirubin is more and more reduced to stercobilin and, to a lesser degree, to stercobilinogen.

In adults all the bilirubin reaching the intestine is reduced by bacteria to stercobilin and its chromogen, unless the intestinal contents are hurried through so rapidly that this reduction is incomplete. Then either bilirubin itself or biliverdin may be excreted. The bilirubin of the fæces may be oxidised to biliverdin on standing in air (*cf* also Chapter XII).

Reference was made on p. 243 to the traces of bilirubin in the very pigmented stools of hæmolytic jaundice. It seems that a little bilirubin may escape reduction owing either to rapid transit or to high concentration, but in practice the important test is that for stercobilin and its chromogen, and not that for bilirubin.

### Tests for Bilirubin

The writer has found Schmidt's sublimate test (oxidation of bilirubin to biliverdin by  $\text{HgCl}_2$ ) of little value.

Gmelin's nitric acid test (*cf* Chapter II) is fairly satisfactory. It may be applied either to a smear of fæces on a white tile or on filter paper, or to a watery extract of fæces as a ring test. The result should be regarded as positive only if a definite green or blue colour is observed.

Van den Bergh's diazo test cannot readily be applied to fæces.

Fouchet's reagent (see Appendix) is the best of all, a thin suspension, about 1 in 20, of the fæces is treated on a porcelain tile, or in a test tube, with an increasing number of drops of the reagent, but not more than an equal volume is required and usually much less. Bilirubin is oxidised in a few seconds to green biliverdin or blue cholecyanin, and is not over oxidised.

### BLOOD (see "Occult Blood")

#### COLOUR

The colour varies with the diet (see table overleaf) and with the proportion of water (diarrhoea, constipation) and of fat. In clinical work various drugs may affect the colour (see table overleaf). In pathological conditions the fæces may be tinged red by unaltered blood, or, in melæna, may be dark to "tarry," or even black, due to altered blood (alkaline hæmatin). They may be darker than usual owing to excess of bile derivatives (*cf* Chapter XII), or paler than usual owing to a deficiency of bile derivatives, or to an excess of fat (*cf* Chapters XII and XIII). Meconium is dark green or blackish green, due to biliverdin. Meconium contains a quantity of porphyrin,

*Influence of Food on Colour of Stool (Hawk and Bergeim)*

Article of Diet	Colour of Stools
Milk . . . .	Light yellow or greyish white
Meat . . . .	Brownish black.
Chlorophyllic vegetables, e.g., spinach . . . .	Greenish
Non chlorophyllic vegetables	Light brown
Cherries or blackberries . .	Reddish brown
Cocoa . . . .	Dark red or chocolate brown
Coffee . . . .	Dark brown
Corn meal . . . .	Light coloured

*Influence of Drugs upon the Colour of the Stool (Hawk and Bergeim)*

Drug	Dose	Colour of Stools
Bismuth subnitrate, grams	5	Black (bismuth suboxide)
Calomel, mgm .	130 to 140	Green
Reduced iron, mgm	65 to 70	Greyish - black, turning darker on exposure to air.
Methylene blue, mgm	130 to 140	Blue, especially after exposure to air
Manganese dioxide, mgm	130 to 140	Dark brown or black
Hamatoxylin, grams	1	Reddish brown
Rhubarb, c c fluid extract	2	Yellow.
Senna, c c fluid extract	4	Dark yellow.
Cambogia, mgm	130 to 140	Dark yellow
Santonin, mgm .	65 to 70	Dark yellow

much more than the trace in adult fæces. The green colour of diarrhoeic stools in infants is due to biliverdin, or possibly also to a green pigment formed by organisms. Infants' stools often turn green on exposure to air owing to oxidation of bilirubin to biliverdin, or possibly in some cases to the formation of green pigment by bacteria.

**"FAT" (NEUTRAL FAT, FATTY ACIDS, SOAPS, LIPOIDS)**

The term "fat" is loosely applied. An estimation of "total fat" includes all substances soluble in ether, together with the fatty acids liberated from soaps by acid. The substances soluble in ether include neutral fat (the triglycerides of oleic, palmitic and stearic acids), free fatty acids, sterols, pigments and minute amounts of other substances that need not be considered. "Split fat" includes preformed fatty acids and fatty acids liberated from soaps. "Unsplit fat" includes neutral fats, sterols, pigments, etc., and is calculated from the difference between the total and the split fat; it will, therefore, include any liquid paraffin or other neutral oily matter which may travel through into the fæces after oral administration. It is essential that liquid paraffin be avoided for three or four days before the collection of the fæces for fat analysis.

Total fat	Split fat	{ Free fatty acids Fatty acids liberated from soaps
	Unsplit fat	{ Neutral fats (triglycerides) Sterols Pigments (traces only) Other substances (minute traces) (Liquid paraffin, etc.)

Analyses may be made on the moist fresh fæces or on the dried fæces. In the former case the ratio of the wet weight to the dry weight must be determined separately, so that the results may be expressed in terms of the dry weight. It would be useless to express the results in terms of the moist fresh fæces owing to the great variation in water content (diarrhoea, etc.). Each method has its protagonists, but the majority of workers prefer to dry the fæces. One objection to the use of undried fæces is that the lower fatty acids, which are derived mainly from celluloses, etc., will be extracted. When fæces are dried, as subsequently described, these lower fatty acids are largely volatilised.

For routine clinical work the method of Holt, Courtney and Fales is recommended. For special investigations, extraction in a Soxhlet apparatus is advised (see later). For details of the different methods available the reader is referred to the list of references. A few special points only will be noted here. In clinical work it is a common practice to heat the fæces on a boiling water bath until it is possible to reduce them to a powder. This takes from a few hours to one or two days. This powder is, of course, not strictly dry. It is necessary to place it in a vacuum desiccator for several days to take it to constant weight. This desiccation is frequently omitted because the clinician cannot afford to wait so long for the analysis. An error of 3 to 10 per cent is thereby introduced.

Hydrolysis of the soaps with acid must not be performed in the presence of alcohol if the fatty acids are subsequently to be determined by titration with alkali, because esterification may occur and introduce a serious error. (The soaps may be hydrolysed with

an aqueous solution of acid, as in the method of Holt, Courtney and Fales Cammidge's method, in which hydrolysis by aqueous acid is also employed, has been shown to be inaccurate owing to incomplete extraction) Alcoholic acid may be adopted, however, when the ether extract is to be weighed (see later under "First Soxhlet method")

If aqueous acid is used for hydrolysis of the soaps in the original dried faeces, the resulting mixture cannot be taken to dryness (and extracted with ether), because a dark tarry mass results which is difficult or impossible to remove from the dish The difficulty may be overcome as described later under "Second Soxhlet method"

When calculating the split fat from the titration of the fatty acids with alkali the factor "1 cc of N/10 alkali corresponds to 0.0268 gm of fatty acid" is recommended

### Interpretation of Results

Analyses of dried stools merely indicate the *percentages* of fat present in those stools Without a knowledge of the exact intake and output of fat, it is impossible to decide how much of the food fat has been digested and absorbed Certain deductions, however, can be made, thus when, on an ordinary diet, the percentage of total fat in the faeces is much higher than normal, it is clear that either digestion or absorption or both must have been deficient In these cases the ratio of split to unsplit fat shows whether it is digestion or absorption that is mainly at fault

**The Normal Findings in Children** The following table is compiled from the extensive data of Holt, Courtney and Fales

It will be seen that the normal varies with age and the type of diet In infants the total fat not uncommonly amounts to 50 per cent of the dried faeces After infancy it rarely exceeds one third

### Differential Estimation of Fat in Faeces of Normal Infants and Children

(Holt, Courtney and Fales)

Age	Diet	Total Fat			Number of Analyses	Percentage of Faecal Fat						Number of Analyses
		Percentage of Dried Faeces				Split			Unsplit			
		Max	Min	Av		Max	Min	Av	Max	Min	Av	
8 days to 10 months	Breast milk	61.1	4.4	34.5	44	92.1	51.7	79.8	48.3*	7.9	20.2	44
2 to 18 months	Cow's milk modifications	89.6†	10.2	34.1	94	97.1	22.7	87.9	77.3‡	2.9	12.1	57
18 months to 10 years	Milk or milk bread and cereal	45.2	17.7	30.7	10	95.1	69.1	86.0	30.9	4.9	14.0	10
1 to 7½ years	Mixed	39.1§	8.7	13.7	6*	89.1	44.0	73.1	56.6	10.9	26.9	8*

\* Next highest 33.2

† Next highest 56.7

‡ Next highest 55.1 and 31.7

§ Next highest 33.7

Only nine exceed 34.0



Of the faecal fat the percentage which is unsplit rarely exceeds one third, both in infants and children. When interpreting results in diseases of children the following generalisation is therefore justified — *Of the dried faeces not more than one third should be fat, and of that (faecal) fat not more than one third should be unsplit, remembering that in infants even one half of the dried faeces may be fat*

**The Normal Findings in Adults** In normal adults the findings are the same as in children except that a slightly smaller percentage of the dried faeces is "fat," and a rather larger proportion of the faecal fat is split. The following generalisation may be safely accepted as a rough guide — *Of the dried faeces not more than one-quarter should be fat, and of that (faecal) fat not more than one-quarter should be unsplit*

In clinical work there is little or no useful information to be gained from separate estimations of free fatty acids and soaps. The ratio of free to combined fatty acids (soaps) depends largely on the reaction of the intestinal contents. The ratio of unsplit to split (*i.e.*, free fatty acids plus soaps) fat is the important factor, because it indicates the efficiency of fat digestion, whilst the total fat gives a measure of the efficiency of absorption.

As already mentioned, the term "unsplit fat" includes neutral fats (triglycerides), sterols, pigments, and minute amounts of other substances which need not be considered. The weight of the pigments is so small that it can be disregarded. In twelve normal adults Fowweather obtained the following results for the unsaponifiable matter (which consists mainly of sterols) — 0.64 to 3.88 per cent of the dried faeces, or 7.8 to 33.3 per cent of the unsplit fat. For most clinical purposes, therefore, the quantity of sterols need not be determined.

In all the above analyses, what has been determined is the percentage composition, the concentration of the fats, in the sample of faeces analysed. It is important to bear this in mind when interpreting results, otherwise misleading deductions may occasionally be made. The position is somewhat similar to the consideration of sugar analyses in urine. The sugar percentage, for instance, might rise, and yet at the same time the day's output might fall owing to the occurrence of oliguria. In the same way the concentration of faecal fat might appear slightly high, and yet the day's output be low, owing to the total daily weight of dried faeces being low.

Another similar point arises in connection with the percentage of the faecal fat which is split or unsplit respectively. The following is typical of cases on low fat diets with normal digestion and fat absorption —

(a) Expressed as percentage of dried faeces

	(1)	(11)
Split fat . . . .	6.4	6.26
Unsplit fat . . . .	3.9	3.44
Total fat . . . .	10.3	9.70

(b) Expressed as percentage of the faecal fat :

	(i)	(ii)
Split	62.1	64.5
Unsplit	37.9	35.5

The figures for total fat in (a) are obviously normal, but in (b), at first sight, the unsplit fat might appear slightly raised. The figures in (b) must, however, be interpreted in the light of the results in (a). When the fat in the diet is low the split fat tends to be almost completely absorbed. The unsplit fat, however, corresponds more or less to "starvation" faecal fat (cf. Hill and Bloor, Fleming and Hutchison). The exact significance of the latter is not entirely settled, but for our present purposes it may be regarded as fatty matter (including sterols) which has either been excreted into the bowel, or has been formed by the degeneration of epithelial cells, or has been derived from the bodies of bacteria, etc. In other words, a proportion of the unsplit fat is not a residue of the food taken in the diet. When the fat intake is very small this non food fatty residue may form a high percentage of the faecal fat.

**Pathological Findings.** Faecal fat percentages give a rough indication of the efficiency of the digestion and absorption of fat. By themselves such estimations are seldom, if ever, pathognomonic of any one disease, though they are often valuable as an extension of the clinical examination in the diagnosis of certain diseases (untreated) and as a guide to treatment by restriction of fat in the diet. Their value in pancreatic disease (Chapter XIII), and the influence of bile on fat absorption (Chapter XII), have already been discussed. Simple diarrhoea may cause deficient splitting and deficient absorption of fats. Chronic intestinal indigestion in children is frequently accompanied by an excess of fat in the stools, and the splitting is generally normal. Indeed, in the more severe forms of this condition the results of fat analyses are identical with those found in typical coeliac disease. In sprue, likewise, the total fat is in excess and the splitting normal. In some cases of tuberculous peritonitis in children similar results are obtained, probably due to obstruction of the lacteals. In the rare condition, congenital steatorrhoea (see Garrod's *Inborn Errors of Metabolism*), the total fat is in great excess, but the degree of splitting varies in the few reported cases. In most cases of rickets the results are within normal limits. In gastroenteritis, and enteritis also, the findings are usually inside the normal range, possibly, in part at any rate, owing to inability to ingest much fat.

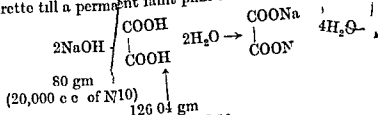
For metabolism experiments, see Chapter XXV.

#### Estimation of Total Fat and of Split Fat by Method of Holt, Courtney and Fales

**Principle.** A weighed quantity of dried faeces is hydrolysed with aqueous hydrochloric acid to convert soaps into fatty acids. Extraction is made with suitable fat solvents, the solvent is removed and the extracted "fat" weighed. It is then redissolved and the

solution titrated with standard alcoholic solution of sodium hydroxide to estimate the "split" fat (fatty acids preformed and liberated from soaps)

**Solutions** (1) *N/10 Alcoholic Sodium Hydroxide* Dissolve 4.2 gm of pure sodium hydroxide in a few cubic centimetres of distilled water and dilute to 1,000 c.c. with absolute alcohol. Stand overnight, filter, and determine its exact concentration by titration with *N/10 oxalic acid* as follows. Place 20 c.c. of *N/10 oxalic acid* in a beaker, add 3 or 4 drops of 1 per cent phenolphthalein indicator, 1 per cent alcohol, and titrate with the alcoholic sodium hydroxide from a burette till a permanent faint pink colour is obtained.



Suppose  $y$  = 20 c.c. of 0.1 *N* alcoholic *NaOH*, which is  $y$  normal = 10

Then

$$\begin{array}{l}
 y \times x = 20 \times 0.1 \\
 \frac{20 \times 0.1}{y} = x
 \end{array}$$

(2) *No Oxalic Acid* Dissolve exactly 0.6302 gm of pure crystalline oxalic acid in about 10 c.c. of distilled water in a 100 c.c. volumetric flask, and make up to volume with water.

**Dry the Fæces** Transfer the sample of fæces (about 50 gm is convenient as a rule) to a large (e.g., 9 or 10 in diameter) evaporating basin. Place on a boiling water bath in a fume cupboard and heat till "dry". Powder with a pestle, add about 50 c.c. of alcohol and stir again. Repeat if necessary until the fæces can be reduced to a fine powder. Reject any coarse lumps of hair, tow, gristle etc. and store in a vacuum desiccator overnight (routine work), or until constant weight is attained (special work).

**Analysis** Take a portion (about 1 gm) of the dried powdered fæces, and determine its weight accurately, using a tared watch glass. Transfer to a small evaporating basin, washing it in with about 10 c.c. of 25 per cent v/v hydrochloric acid. Heat on a boiling water bath until the contents of the dish are thoroughly disintegrated (about twenty minutes), but avoid evaporation to dryness.

Cool, and wash quantitatively into a Roehrig tube (Fig 80) with distilled water up to the mark " $\frac{1}{2}$ ". Add alcohol (absolute or 95 per cent) to the "0" mark (level of tap). Make sure that the tap is closed and add pure ethyl ether up to the 25 c.c. mark. Introduce the glass stopper securely and shake vigorously for half a minute. Add light petroleum ("petroleum ether"), of boiling point lower than  $60^\circ\text{C}$ , up to the 50 c.c. mark, and shake again for half a

minute. Allow to stand until the emulsion separates. This is facilitated by cautiously rotating the tube in a vertical position from time to time. Run off the ether through the tap on to a rapid fat free filter (No. 43 Whatman) placed in a very light previously weighed flask of about 200 c c capacity (Fig. 80).

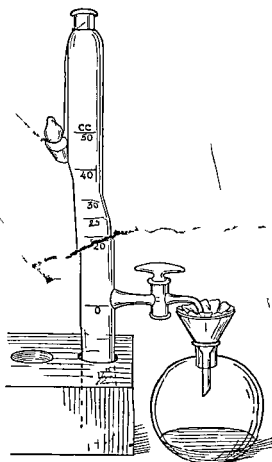


FIG. 80. Roehrig tube as used for faecal fat analyses. It is made out of tubing 1 to  $1\frac{1}{2}$  in. in diameter. Height without stopper about 12 in. Capacity up to  $\frac{1}{2}$  mark about 20 cc. up to 0 mark about 40 cc.

Close the tap and perform a second extraction with 20 cc of ether plus 20 cc of light petroleum. Separate and pass the ether layer through the same filter. Make a third and last extraction with 30 cc of "recovered" mixed ether plus light petroleum.

Finally wash the nozzle of the tap and the filter paper with about 10 cc of the recovered solvents.

Fit an ordinary cork carrying a bent tube (as air condenser) into the flask, and distil off the mixed solvents on a water bath. This takes only a few minutes. Store the recovered solvents for future use.

Clean thoroughly the outside of the flask and place it in a

vacuum desiccator ( $\text{CaCl}_2$  plus paraffin wax shavings) overnight (routine work) or until constant weight is attained (special work)<sup>1</sup>

Determine accurately the weight of the flask with its contents, and hence by difference the weight of the total fat

Redissolve the fat by adding about 50 c c of pure benzene ( $\text{C}_6\text{H}_6$ ) and heating almost to boiling on a water bath. Titrate whilst hot with N/10 alcoholic sodium hydroxide, adding about  $\frac{1}{2}$  c c of 1 per cent phenolphthalein in 50 per cent alcohol as indicator. The end point is shown by the appearance of a faint pink colour which persists for thirty seconds or more.

Calculate the split fat from the factor 1 c c of N/10 alcoholic  $\text{NaOH} = 0.0268$  gm of mixed fatty acids, and the unsplit fat by difference (total - split fat)

Express the total fat, split fat and unsplit fat as percentages of the dried fæces, and the split and unsplit fat as percentages of the total fat, as illustrated in the example which follows

**Example of Calculation :—**

(a) Weight of dried fæces	= 1.0 gm
Weight of flask and fat	= 15.8736 gm
Weight of flask	= 15.5602 gm
Weight of fat	= 0.3134 gm

1 gm of fæces contains 0.3134 gm of fat  
 100        "        "        31.34        "

Total fat = 31.34 per cent

(b) The 0.3134 gm of fat was redissolved and required 4.5 c c of 0.1 N alcoholic  $\text{NaOH}$  for neutralisation

Since 1 c c of 0.1 N alcoholic  $\text{NaOH}$  is equivalent to 0.0268 gm of mixed fatty acids,

4.5 c c of the  $\text{NaOH}$         =  $0.0268 \times 4.5$  gm of fatty acids,

1 gm of dried fæces contains         $0.0268 \times 4.5$  gm of fatty acids,

• 100        "        "        "         $0.0268 \times 4.5 \times 100$  gm fatty acids

These fatty acids were partly preformed and partly liberated by  $\text{HCl}$  from soaps,

i.e.,        Split fat =  $2.68 \times 4.5 = 12.06$  per cent

Therefore, by difference the unsplit fat is  $31.34 - 12.06 = 19.28$  per cent

<sup>1</sup> Alternatively an unweighed flask may be used. In that case the residue after removal of solvent is extracted with 3 or 4 portions each of about 10 c c of light petroleum which are filtered into a weighed flask. The solvent is removed as described from this second flask, which is then cleaned externally, desiccated and weighed as indicated above.

(c) Since, out of 31.34 gm of faecal fat 12.06 is split,

" 1 " "  $\frac{12.06}{31.34}$  is split,

" 100 " "  $\frac{12.06 \times 100}{31.34}$  is split,

i.e., split fat is 38.48 per cent of faecal fat,

. unsplit " 61.52 " "

(d) Report as follows —

Unsplit (neutral) fat	= 19.3	} per cent of dried faeces,
Split fat	= 12.0	
Total fat	= 31.3	

and, of the faecal fat 38.5 per cent is split

61.5 " unsplit

**Notes** A separate determination of soaps may be made, if desired, by digesting 1 gm of dried faeces with 10 c.c. of water, instead of 10 c.c. of hydrochloric acid. The suspension is transferred to a second Roehrig tube and treated throughout as above. The ether extract will then contain neutral fat plus preformed fatty acids. The soaps are calculated by the difference in weight between the ether extracts of the first and of the second tube respectively. The ether extract of the second tube is redissolved and titrated to obtain the preformed fatty acids. The neutral fat is calculated by difference.

The writer does not recommend this procedure, because the emulsion in the second tube (no acid) frequently will not separate satisfactorily and because a separate determination of soaps adds little or nothing of value clinically.

The stopper and tap of the Roehrig tube may be lubricated with glycerol, or may be used without a lubricant, naturally vaseline or tap grease must be avoided.

### Estimation of Liquid Paraffin

As previously mentioned, liquid paraffin and oily drugs should be omitted for three or four days before collecting the faeces for fat analysis, but it is sometimes desirable in the laboratory to test whether this precaution has been taken. The following procedure, for drawing my attention to which I am indebted to Dr H. E. Archer, may be utilised —

Repeat the estimation of fat in dried faeces as previously described up to the stage when the weight of ether extract ("fat") has been determined. Then instead of redissolving the fat in benzene, add cautiously about 5 c.c. of concentrated sulphuric acid to the flask. The acid will destroy the fats and fatty acids, but will not affect liquid paraffin. Heat well almost to boiling to complete the decomposition. Cool well and pour slowly and carefully into about 50 c.c. of water in a separating funnel. Cool the funnel thoroughly. Wash out the beaker with water, then with light petroleum, and add

the washings to the funnel. Add 20 to 30 c c of petroleum and shake well. Stand and remove the petroleum layer. Make a second extraction with 10 to 15 c c of petroleum. Combine the extracts in a weighed light flask. Distil off the petroleum, dry and weigh in the usual way.

Calculate the quantity of liquid paraffin per 100 gm of dried fæces, and correct the neutral fat figure

Example —

$$\text{First analysis} \left\{ \begin{array}{l} \text{Neutral unsplit fat} = 41.3 \\ \text{Split fat} = 23.5 \\ \text{Total fat} = 64.8 \end{array} \right\} \text{per 100 gm of dried fæces}$$

And, of the fæcal fat 36.3 per cent is split, and 63.7 per cent is unsplit

By the second analysis 33.1 gm of liquid paraffin were found per 100 gm of dried fæces. Therefore the corrected neutral fat is  $41.3 - 33.1 = 8.2$  gm per cent. The corrected report therefore reads —

$$\left. \begin{array}{l} \text{Neutral unsplit fat} = 8.2 \\ \text{Split fat} = 23.5 \\ \text{Total fat} = 31.7 \end{array} \right\} \text{per 100 gm of dried fæces}$$

And of the fæcal fat 74.1 per cent is split, and 25.9 per cent is unsplit.

#### Estimation of Total Fat and of Split Fat using a Soxhlet Apparatus

Continuous extraction in a Soxhlet apparatus (Fig 66, p 350) is employed instead of three simple extractions, but otherwise the method is substantially the same as that of Holt Courtney and Fales described above. The same reagents (p 465) and the same technique for drying the fæces (p 465) are used.

**First Method Separate Determination of Soaps** Weigh a Soxhlet thimble. Add about 1 gm of the dried fæces and weigh again. The difference gives the exact weight of the fæces. Transfer to the Soxhlet apparatus, and extract continuously with ether for at least six hours. Distil off the ether and extract the residue with three or four portions of light petroleum each of about 10 c c, filtering into a second weighed light flask, through a fat free paper. Distil off the solvent, desiccate overnight (routine work) or to constant weight (special work) and weigh. The weight, less that of the flask gives the amount of neutral fat plus free fatty acids in the sample taken. Calculate the amount in 100 gm of dried fæces.

Dissolve in hot benzene and titrate with 0.1 N NaOH and phenolphthalein (cf p 467). Calculate the free fatty acids per 100 gm of dried fæces.

Remove the thimble which contains the soaps and other matter insoluble in ether, and set aside for any residual ether to evaporate. Transfer the contents of the thimble quantitatively to an evaporating basin, and add 10 c c of 10 per cent HCl in absolute alcohol. Digest

on a boiling water bath till a thin syrup is obtained. Take up the syrup with plaster of Paris or anhydrous sodium sulphate, and return quantitatively to the thimble, which is placed in the Soxhlet apparatus and extracted with ether as before. Remove the solvent from the weighed flask, dessicate and weigh as usual. The weight, less that of the flask, gives the soaps. Calculate for 100 gm of dried faeces

The digestion with 10 c.c. of 10 per cent alcoholic HCl liberates fatty acids from the soaps. Some of the fatty acids combine with alcohol to yield ethyl esters. It is owing to this partial esterification that the ether extract must be weighed and not titrated. The degree of esterification depends mainly on the duration of heating. The maximum error which could be introduced by substitution of the ethyl group for hydrogen is of the order of + 10 per cent.

**Example of Calculation :—**

Weight of dried faeces = 1.0 gm  
 Weight of first extract = 0.235 gm  
 Therefore neutral fat + fatty acids = 23.5 per cent

First extract required 50 c.c. of 0.1 N alcoholic NaOH, and therefore contained  $0.0268 \times 50$  gm of fatty acids

Therefore free fatty acids =  $0.0268 \times 50 \times 100$ , or 13.4 per cent and neutral fat =  $23.5 - 13.4$ , or 10.1 per cent

Weight of second extract = 0.285 gm

Therefore soaps = 28.5 per cent

The completed result therefore is —

Neutral (unsplit) fat	10.1	} per cent of dried faeces,
Free fatty acids	13.4	
Soaps	28.5	
Total fat	52.0	

and, of the faecal fat 19.4 per cent is unsplit

80.6 " " is split

**Second Soxhlet Method : Determination of Total and of Split Fat** 1 gm of dried faeces and 10 c.c. of 25 per cent v/v aqueous HCl are digested exactly as in the method of Holt, Courtney and Fales (p. 465). Plaster of Paris or anhydrous sodium sulphate is added to obtain a free flowing powder, which is transferred quantitatively to a thimble and extracted with ether for at least six hours in a Soxhlet apparatus. The ether is removed and the residue dissolved in petroleum ether and transferred to a weighed flask as described in the first Soxhlet method. The solvent is removed and the residue is desiccated and weighed as usual. From this is calculated the total fat per cent.

The fat is then dissolved in hot benzene and titrated with alcoholic NaOH to obtain the split fat

**Example of Calculation :—**

Weight of dried faeces = 1.0 gm  
 Weight of extract = 0.58 gm

Total fat is 58.0 per cent

Titration of split fat = 17.9 c.c. of 0.1 N alcoholic NaOH



∴ Splt fat  $0.0268 \times 17.9 \times 100$ , or 48.0 per cent

∴ Unsplt fat =  $58.0 - 48.0$ , or 10.0 per cent

Neutral unsplt fat	= 10.0	} per cent. of dried fæces,
Splt fat	= 48.0	
Total fat	= 58.0	

and, of the faecal fat 17.2 per cent is unsplt,  
82.8 „ „ is splt

**Notes** The top of the thumble should be a few millimetres above the top of the syphon of the Soxhlet apparatus. If it does not reach so high, pack a piece of screwed up fat free filter paper underneath it.

Introduce into the mouth of the thumble another piece of screwed-up fat free paper, to prevent drops of condensed solvent splashing particles of powder out of the thumble.

In the second Soxhlet method some samples of fæces yield a solution in hot benzene so pigmented that it is difficult or impossible to titrate it. Continuous extraction, though it takes out 100 per cent of the fat, has the disadvantage that it also extracts pigments to a much greater extent than the three simple extractions in the method of Holt, Courtney and Fales.

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 HUTCHISON, H. S. *Quart J Med*, 1920, 13, 277.  
 LAWS, C. H., and BLOOR, W. R. *Amer J Dis Child*, 1916, 11, 229.  
 TIDWELL, H. C., and HOLT, L. E. *J Biol Chem*, 1936, 112, 605 (A good method but requires a 50 c.c. centrifuge tube).

### MUCUS

An excess of mucus is often recognisable by inspection of the fæces with the unaided eye. It may be roughly separated by shaking a portion of the stool with water, and allowing the coarse particles of undigested food, foreign bodies, etc., and mucus to settle by gravity. Under the microscope it appears as shreds or streaks, which often show lines on treatment with dilute (e.g., 1 per cent) acetic acid. Mucus is soluble in alkali, and insoluble in acetic acid, and is thrown out from a faecal suspension in water.

by protein precipitants, e.g., alcohol. In clinical work chemical tests are unnecessary.

Mucus is always being secreted by the healthy mucous membranes of the alimentary tract, but not in quantities sufficient to be obvious to the naked eye. An excess of mucus in the faeces is the rule in inflammatory conditions of the gastro intestinal canal, and especially in colitis. If mucus is obvious macroscopically the probability is that it is derived from the colon. If the microscope is required for its detection it is more likely to have come from the small intestine. If the faeces are strongly alkaline, mucus may pass into solution. Bacteria may digest, or render unrecognisable, small quantities of mucus.

### NITROGEN

The nitrogen of the faeces is derived from the food proteins, the secretions and excretions of the alimentary canal, and the bacteria. In starvation the faeces contain nitrogen, about 0.25 gm. daily. On non nitrogenous diets the faecal nitrogen is increased, probably due to the increase of alimentary secretions, of bacteria, etc., but it still amounts to only about 0.75 gm. On a full mixed diet the faecal nitrogen again increases, but only slightly, to about 1 gm. per diem in health. In other words, the food proteins are normally almost completely digested and absorbed.

In disease the faecal nitrogen may be considerably increased owing to failure of protein digestion, or to incomplete absorption of the products of digestion. The subject has already been mentioned in Chapter XIII, where it was concluded that nitrogen estimations are of little value clinically owing to the variety of diseases which may cause an increased loss in the faeces. The fact that nitrogenous food is being undigested or lost in the faeces can be deduced by simpler methods than nitrogen analyses.

In metabolism experiments on subjects whose faeces show no evidence microscopically of excessive loss of nitrogen containing residues from food it is a common practice to allow 1 gm. for the faecal nitrogen daily, but obviously when there is such evidence the nitrogen must be estimated. Peters and Van Slyke (*Quantitative Clinical Chemistry*, Vol. II) advise against drying the faeces, and recommend the following procedure to prevent loss of ammonia. The day's stool is mixed well with about 300 c.c. of water, and 250 c.c. of concentrated sulphuric acid are added slowly with constant stirring so that a homogeneous black suspension is obtained. The mixture is cooled, treated with a few drops of caprylic alcohol to prevent frothing, and diluted with water to 1,000 c.c. After mixing thoroughly, portions of 25 c.c. may be used for the macro Kjeldahl technique, 25 c.c. of 0.2 N sulphuric acid being used to catch the ammonia.

### OCCULT BLOOD

Blood may gain access to the stools from any part of the alimentary tract from the mouth to the anus. Blood added at the anus

simply means that there is a hæmorrhage somewhere between mouth and anus. It is, therefore, essential to inspect the mouth, gums and throat, to examine for hæmorrhoids, to inquire about the menses, etc., before the significance of positive tests can be assessed. In other words, when the chemical pathologist reports that blood is present in the fæces, the clinician decides as to its significance.

The tests are simple and are often valuable in clinical work, both as an aid to diagnosis and in following the results of treatment of ulcers. Thus in suspected ulcers (stomach, duodenum, small intestine, colon, etc.) it is important to test the fæces for blood. Hæmorrhage is often intermittent, hence repeated tests are valuable. Obviously a negative result does not necessarily mean there is no ulcer—the ulcer may not be bleeding at the time of the test. Obviously, also, a positive test gives no indication of the type of ulceration, whether simple or malignant, whether tuberculous, and so on. The tests are particularly valuable in following the progress of proved cases of gastric and of duodenal ulcer. It would be unwise, for instance, to allow such a patient to return to a full diet before the tests for occult blood have become negative. It is possible for the patient to feel perfectly well, although there is still slight hæmorrhage from the ulcer, as shown by positive tests for occult blood in the fæces.

Ulcers and hæmorrhages from new growths are the commonest causes of occult blood in the stools, but any type of hæmorrhage may be responsible, *e.g.*, in acute hæmorrhagic pancreatitis, ruptured œsophageal veins in cirrhosis of the liver, oozing of blood in pernicious anæmia, in blood diseases and hæmorrhagic conditions, and in scurvy, etc., etc. It is necessary to take  $\frac{1}{2}$  to 1 c.c. of blood by mouth in order to produce a positive benzidine test in normal fæces and 1 c.c. or more to produce a positive spectroscopic test (Abrahams).

**Consideration of Methods.** It is wise to make it a routine practice to examine microscopically a suspension of the fæces in 0.65 per cent. NaCl (*cf.* p. 455), so as to detect red cells and muscle fibres (see above). Otherwise the tests fall into two main groups: (a) the catalytic effect of iron containing blood derivatives on the interaction of hydrogen peroxide and certain organic substances (benzidine, guaiac, pyramidone) to yield coloured oxidation products, and (b) the extraction of blood derivatives with special solvents, and spectroscopical examination of the solutions. In certain methods the extracts are treated to yield other hæmoglobin derivatives, and their characteristic spectra are looked for.

As an example of group (a), the benzidine test will be considered more fully. Hydrogen peroxide alone will not oxidise benzidine to the blue oxidation compound. The reaction will only take place in the presence of a catalyst, which may be a peroxidase or an iron containing derivative of hæmoglobin. Porphyrin, which is iron free, will not catalyse this reaction. It is, therefore, possible for the fæces to contain porphyrin, and yet to give a negative

**benzidine test** Fæces often contain thermolabile enzymes which will act as catalysts. It is, therefore, essential to boil the fæces so as to destroy these enzymes. The catalytic action of the blood derivatives is unaffected by boiling.

Normal fæces do not show the bands of hæmatin, but those of porphyrin may be seen with certain techniques in a proportion of healthy subjects, thus some workers for this reason attach little significance to a positive finding for porphyrin by Snapper's method.

When the bleeding is low in the gut a suspension of fæces ground up in water, and cleared by centrifuging, shows the bands of oxyhæmoglobin when examined spectroscopically, when viewing a layer 1 in thick, a suspension of 1 gm of fæces in 40 c c is usually suitable after centrifuging. This method is not widely employed but would be useful, for instance, as a check on Snapper's method, thus by comparing the oxyhæmoglobin content with the acid hæmatin content as determined by Snapper's method, it is possible to judge approximately how much of the acid hæmatin was formed from oxyhæmoglobin during the test, and how much from alkaline hæmatin passed as such in the fæces.

In disease, the higher in the gut is the hæmorrhage, and the slower the passage of fæces, the greater is the chance of porphyrin being formed. Anal bleeding does not give rise to porphyrin in the fæces. As already mentioned, porphyrin does not give a positive benzidine test. The spectroscopic test for hæmatin is not so sensitive as the benzidine test. Even if the patient is not on a hæmoglobin free diet, the finding of hæmatin on spectroscopic examination of the fæces signifies "true" bleeding.

Bloem found the following to be the limits of detection for different methods, the spectroscopic examinations being made with 1 in layers of solution (see his paper for details). Clearly the benzidine test is much more sensitive than the others.

Substance	Absorption Bands	Technique	Limit of Detection mgm. (as $1/6\text{O}_2$ ) per 100 gm fæces
Red blood cells	—	Microscopical	120
Oxyhæmoglobin	$\alpha$ and $\beta$	Spectroscopic	360
Hæmoglobin and/or hæmatin.	—	Benzidine (hæmoglobin free diet)	1.6
Alkaline hæmatin	$\alpha$	} Snapper's test	440
Alkaline porphyrin	$\alpha$ and $\beta$ and $\gamma$		200
Hæmochromogen	$\alpha$ and $\beta$		65
Acid porphyrin	$\alpha$ and $\beta$		140
Acid hæmatin	$\alpha$ and $\gamma$		200

**Information from a Combination of Tests** Reference has been made in previous paragraphs to some points in this connection, but they may usefully be summarised and extended. The site of the hæmorrhage may be gauged approximately by a combination of the microscopical, spectroscopical and catalytic tests. Thus red cells must be derived from contamination, the anus or rectum,

unless the hæmorrhage be gross when the colon might be the site. When the patient has not been prepared (p 473) a few red cells may sometimes be observed microscopically, although the benzidine test is negative, which may perhaps be explained by Barrett's observations (see p 473). Oxyhæmoglobin in a cleared faecal suspension likewise implicates the lowest parts of the alimentary canal. Porphyrin,<sup>1</sup> in excess of possibly normal traces, indicates hæmorrhage in the upper part of the gut, for it is unlikely that it can be formed from blood shed below the colon. Naturally, in attempting these interpretations, the degree of bleeding and the rate of passage of intestinal contents must also be taken into consideration.

Some idea of the size of the hæmorrhage can be obtained by noting the intensity of the positive reactions, and from a knowledge of the relative sensitivity of the tests (see table on p 475). It is a good plan to perform the pyramidone test whenever the benzidine reaction is positive. The pyramidone test is the less sensitive of the two so that if both are positive there is a greater concentration of blood than if the benzidine test is positive but the pyramidone test is negative. In following the progress of hæmorrhage (from ulcer) it is useful to note changes from "pyramidone and benzidine positive," to "benzidine positive pyramidone negative," to "benzidine negative." Both tests, however, are much more sensitive than the spectroscopic test for hæmatin, so if the latter is negative bleeding is slight.

#### Technique of Tests for Occult Blood

**Benzidine Test** Place a portion of the faeces about the size of a pea in a boiling tube and add about 10 c.c. of distilled water. If the faeces are liquid, dilute less or not at all. Boil well to render oxidising enzymes inactive, and cool. In another perfectly clean boiling tube place a large "knife point" of benzidine and 2 or 3 c.c. of glacial acetic acid. Shake well to obtain a saturated solution. If all the benzidine dissolves, add a trace more. A few specks of undissolved benzidine do not interfere, but if the solution is not saturated the test is not so sensitive. Add an equal volume of "ten volumes" hydrogen peroxide and shake. Watch the benzidine peroxide solution for a few minutes to see whether a green or blue colour develops owing to the tube not being really clean. The writer has obtained no false positives since adopting as a routine the cleaning of all test tubes with tap water, boiling 10 per cent washing soda ( $\text{Na}_2\text{CO}_3$ ), tap water, commercial hydrochloric acid (about 5 per cent v/v), tap water, and, lastly, three portions of distilled water (see Appendix).

Add the faecal suspension to the benzidine peroxide solution drop by drop, shaking after each addition and noting any colour change. If necessary add altogether an equal volume of the faecal

<sup>1</sup> Stercoporphyrin due to the very rare condition congenital porphyria, or in certain cases of *hydrops aestivale* unaccompanied by porphyrinuria, has also to be considered (see Chapter X).

suspension. A green or blue colour, which often develops rather slowly, results if blood is present. The quantity of faecal suspension required, and the rate of development and intensity of the blue colour, gives a rough measure of the concentration of blood.

When positive results are obtained, make sure that the patient has received a hæmoglobin free diet for three days, and examine microscopically another portion of the fæces for partially digested muscle fibres and for red cells (see above).

**Gregerson's Slide (Benzidine) Test.** Powders, each containing 0.2 gm. of barium peroxide and 0.025 gm. of pure benzidine, are put up in waxed papers.

Dissolve one powder in 5 c.c. of 50 per cent. acetic acid.

Make a smear of the fæces on a slide and add a few drops of the solution. A blue or green colour developing within thirty seconds is regarded as positive. If the colour develops after thirty, but within sixty seconds, the result is regarded as suspicious. Any colour developing after sixty seconds is disregarded.

It has been claimed that no diet restriction is necessary with this procedure, though Ogilvie notes that the ingestion of bone marrow gave a positive result in one case. It will be observed that the fæces are not boiled to render oxidising enzymes inactive. The writer has compared the two methods on a series of fæces, and greatly prefers the first. With the slide method there has often been difficulty in deciding whether a green tinge has definitely developed within thirty seconds, whereas the colour changes in the test tube method are clear-cut. In the second method the technique is made less sensitive to avoid the preparation of the patient essential in the first method. In common with others, the writer has sometimes found the slide method negative when the test tube method has given definitely positive results which have been proved to be significant. His conclusion is that the first is the method of choice but that the second is a useful bedside test of more limited value, which should be employed only when the proper laboratory facilities for the first method are not available.

**Spectroscopic Test (cf. Ryffel and Payne).** Place a portion of fæces the size of a large bean in a glass stoppered boiling tube. Add about 10 c.c. of distilled water and mix well with a glass rod. (In the case of liquid fæces, add little or no water.) Add rather more than an equal volume of glacial acetic acid and again mix. Add 1 volume of ether fully equal to the volume of the mixture, and shake thoroughly. Then add water, a few cubic centimetres at a time, to separate the ether. If separation does not occur, repeat the test using a larger volume of ether. Fæces rich in mucus or liquid paraffin are very apt to give an emulsion from which the ether cannot be separated. In such cases the cautious addition of glacial acetic acid should be tried.

Examine the ether layer with the spectroscope in case the bands of all alano porphyrin (p. 212) are visible. But even if they are not seen, remove the ether layer to another boiling tube and add to it about 10 c.c. of hydrochloric acid (25 per cent. v/v). Mix

well by pouring from one boiling tube to another repeatedly. Allow to separate, and examine each layer with the spectroscope.

The upper ether layer may contain acid hæmatin (p 212) and chlorophyll (p 220). The lower watery layer may contain acid (proto) porphyrin (p 212). There will probably be stercobilin (p 220) in both layers.

**Snapper's Method** In a mortar grind up about 10 gm of fæces with about 40 c.c. of acetone. Filter on a Buchner funnel, and wash the precipitate with acetone. Maintain suction till the precipitate is fairly dry. Transfer the precipitate to another mortar and grind it up with 25 c.c. of a mixture of glacial acetic acid, 1 part and ethyl acetate 2 parts. Filter. Between 12 and 20 c.c. of filtrate are usually obtained. Examine this filtrate spectroscopically for alkaline hæmatin and alkaline porphyrin (p 212). Then divide it into two portions.

To the first portion add at least half its volume of pyridine and a few drops of fresh yellow ammonium sulphide. Invert once only to mix and examine with the spectroscope for the bands of hæmo chromogen (p 212).

To the second portion add about half its volume of 10 per cent v/v HCl, and an equal volume of ether. Shake well and allow to separate. Examine the watery layer for the absorption bands of acid porphyrin (p 212).

**Pyramidone Test** Prepare a fæcal suspension as described under 'Benzidine Test,' and with this perform the pyramidone ring test as described for urine (Chapter II)\*.

**Test for Porphyrin only** Mix thoroughly about 1 gm of fæces in 10 c.c. of 1 per cent HCl in alcohol, and leave overnight. Filter and examine spectroscopically for acid porphyrin.

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 SNAPPER I and VAN CREVELD S. *Ergeb d inner Medizin u Kinderheilk*, 1927 32 1 (good bibliography) and *Nederland Tydschr Geneesk* 1927, 2, 401 and 525

### PORPHYRINS

For 'hæmatoporphyrin' (protoporphyrin) see under "Occult Blood," and for coproporphyrin (stercoporphyrin), see Chapter X. For the relationships of the different porphyrins, see Chapter XVIII.

### PROTEIN (COAGULABLE PROTEIN)

Coagulable protein is usually derived from the blood serum of a hæmorrhage or from a serous exudate into the alimentary canal. Protein in the upper part of the gut is rapidly digested so that protein in the fæces generally indicates a hæmorrhage or serous exudation into the large intestine or below, though it may be due

to a big exudate or bleeding higher up. It occurs notably in cases of ulcer or carcinoma of the colon. On rare occasions, and particularly in children, when there is severe diarrhoea, it may even be derived from the food.

#### Method for Detection (Coope, R, *Lancet*, 1923, 1, 1311)

Emulsify thoroughly about 20 gm of fæces in about 40 c c of distilled water at 50° C. Add an equal volume of saturated ammonium sulphate, mix well and filter through a pleated filter paper, repeatedly if necessary, until a perfectly clear filtrate is obtained. If the original fæces is fluid use little or no water.

Five to 10 c c of filtrate are tested for albumin by heat coagulation, or with salicyl sulphonic acid (cf Chapter II). The reaction is positive only when a definite turbidity develops. Colour changes are of no significance. Globulins and mucus are precipitated by the ammonium sulphate. The fact that globulins are not included in the above test does not matter, since they must rarely, if ever, be unaccompanied by albumin in the fæces.

#### REACTION

In clinical work this is usually taken with litmus paper. Pieces of red and of blue litmus paper are moistened with distilled water and rubbed on the fæces and any colour changes noted. In normal adults the reaction is generally alkaline or neutral to litmus. In diseases in which fat splitting is normal, but absorption of fats is defective (see under "Fats"), the reaction is often acid. Acid stools are also the rule when there is excessive fermentation of carbohydrates.

The reaction of the fæces probably depends mainly on the type of diet, both in health and in disease. In general, on mixed and on meat diets the reaction tends to be alkaline, and on a predominantly carbohydrate or fat rich diet, acid. The reaction will also be influenced by the secretions into the gut, by the type of bacterial flora, the rate of passage of the intestinal contents, etc. Indeed, from his own observations, and after studying the reported findings, the writer's impression is that such large variations are observed in health and in different examples of any one disease, that determinations of the reaction are of very limited value clinically. This is reflected in the infrequency nowadays of requests to test the reaction. Obviously the test is of no value unless performed on fresh fæces.

Attempts have been made to place the subject on a firmer basis by measuring the actual pH of the fæces. Such determinations are only possible in well equipped institutions, and as yet are not sufficiently extensive to justify general conclusions. As an example of the importance of such quantitative measurements, Redman's work (*Biochem J*, 1928 22, 15) on the pH of the fæces in rickets may be mentioned. She found that there was no significant difference between the variations in health and the variations in rickets without or with treatment, and was unable to show



in children the shift in reaction from alkaline to acid which has been observed in rats when vitamin D is added to a rickets producing diet. That the measurements are unlikely to be of much value clinically until further investigations have been made is shown by the great variations reported in health, viz, pH 4.6 to 8.8 —

- HOWE P E and HAWK P B *J Biol Chem*, 1912, 11, 129  
pH (electrometric) 7.01 to 8.77
- NELSON C F and WILLIAMS J L *J Biol Chem*, 1916-17, 28, 271  
pH (colorimetric) 4.70 to 7.48 (five males ages 13 to 70)
- RETTGER L F and CHAPLIN, H A, 1921 (quoted by Robinson)  
pH (colorimetric) 5.0 to 6.8
- ROBINSON C S *J Biol Chem* 1922 52, 445  
pH (electrometric) 7.0 to 7.5 (healthy men)
- REDMAN T *Biochem J* 1928 22, 15  
pH (electrometric) 5.3 to 8.6 (normal children)
- TISDALL, I F and BROWN, A. *Amer J Dis Child*, 1924 27, 312  
pH 4.7 to 5.1 in breast fed infants  
pH 4.6 to 8.3 in artificially fed infants
- LUNDING N C *Acta med Scand*, 1928 68, 97  
pH 6.0 to 8.0

### SOLIDS

Like the amount of fresh faeces, the percentage and day's output of dry matter vary considerably, and are of little importance clinically. The following are taken from Cammidge's and Hawk and Bergeim's books —

Amount of solids in 24 hours on mixed diet, 25 to 45 gm			
"	"	"	on vegetable diet, up to 75 gm
"	"	"	fasting, 2 gm (Breithaupt)
			3.4 gm (Cetti)

#### *Influence of Diet on Faecal Dry Matter (Schmidt and Strasburger)*

Diet	Dry Matter per cent
Milk { Nursing infant	15.0
Adult	28.0
Meat	29.0
Bread	25.0
Potatoes	15.0
Cabbage	4.4
Mixed diet	26.0

The weight, when dried, of bacteria excreted by a normal adult is about 8 gm daily

### STERCOBILIN

Stercobilin and stercobilinogen have been fully discussed in Chapter XII. It is here only necessary to describe clinical tests for their detection in faeces.

In Schmidt's test a piece of faeces the size of a walnut is rubbed up with a small quantity of a saturated mercury perchloride solution,

and allowed to stand overnight. The reaction proceeds more quickly at 37° C. A deep red or pinkish red colour develops owing to the formation of a compound of stercobilin and  $\text{HgCl}_2$ . In the writer's experience this test is often unsatisfactory. The following is recommended as simple and reliable —

#### Extraction with Acid Alcohol and Spectroscopic Examination

In a boiling tube place a portion of the feces about the size of a walnut and about 15 c.c. of acid alcohol (1 c.c. of concentrated hydrochloric acid 100 c.c. of absolute alcohol). Shake thoroughly or mix well with a glass rod. Stand for a few hours or overnight if necessary. Filter and examine the filtrate spectroscopically for the characteristic absorption band of stercobilin (Fig. facing p. 220) or by means of Schlesinger's test (Chapter II).

In normal feces a deep brown filtrate may readily be obtained in half an hour or so, and dilution will be necessary to obtain a good view of the absorption band. The undiluted filtrate will block out a large portion of the sun's spectrum. In feces with a subnormal content of stercobilin the extract will be less dark, and dilution will have to be less or may be unnecessary to obtain a good band. When stercobilin (and its chromogen) is practically absent the extract is pale yellow or almost colourless, and the band is only just evident or absent.

The hydrochloric acid converts stercobilinogen into stercobilin, so that the test reveals the presence of both. This is an advantage since both have the same significance. In the writer's experience the above technique is more satisfactory than extraction with either amyl alcohol or chloroform followed by treatment with acid. With each of these solvents an emulsion often forms which is difficult to separate, and extraction would not appear to be so complete as with acid ethyl alcohol. Pathological feces may contain more chromogen than pigment, so that extraction without acidification may be definitely misleading.

For references to other methods the reader is referred to Chapter VII.

#### TRYPSIN

The significance of tests for trypsin has been discussed in Chapter XIII.

The author's attention was drawn to the following simple modification of Kmaskof's gelatin method (*Med. Abn.* 1911, p. 108) by the late Dr. W. d'Este Emery, with the information that it had been published in Germany, but the writer has been unable to discover the publication. The method may be applied equally well to duodenal fluid (see Chapter XXIII) to discharges (subsequent to laparotomy) suspected to contain pancreatic juice, and to gastric juice after adjustment of reaction. It is a test for a protein-splitting ferment which acts in an alkaline medium, and is attractive in that a short incubation period (fifteen to thirty minutes) gives a clear cut result, thereby reducing to a minimum

the influence of digestion by bacteria. The faeces must be fresh, preferably not more than an hour old, since the enzyme activity rapidly deteriorates.

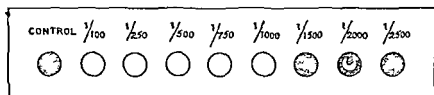


FIG 81 X ray or photographic plate method for trypsin

### The X-ray or Photographic-plate Method

Make a suspension (about 1 in 10 to 1 in 20) of the faeces in 1 per cent crystalline sodium carbonate. Place 1 drop of the suspension and also 1 drop of the sodium carbonate (control) on the film side of a strip cut from an X-ray or photographic plate, and incubate for thirty minutes at 37° C. During incubation, in order to prevent evaporation of the drops, the strip of X-ray plate is placed on a piece of moistened filter paper or blotting paper resting on the perforated shelf or floor of the incubator, and is covered with the lid of a Petri dish or any other suitable cover.

Remove the strip from the incubator, and allow to cool to room temperature, or run water over the reverse surface (no film), so that the gelatin sets properly. Then flood the film with water from the cold tap. If trypsin is present the gelatin is digested to water soluble products, with the result that a clear punched out hole is made in the film. The control (sodium carbonate) area may show a heaping up of the film, but is not dissolved at all. If trypsin is absent the test area has the same appearance as the control area (see Fig 81). A roughly quantitative determination may be made by putting up serial dilutions (see Fig 81).

In the case of duodenal contents and of fluid from an abdominal wound, the pH is usually close to the optimum for the enzyme (pH 8.1) and dilution with sodium carbonate is unnecessary. Gelatin films cannot be used as substrate for the detection of pepsin, because HCl alone causes hydrolysis at 37° C. Before testing for trypsin (due to regurgitation) in gastric contents the reaction must be adjusted with alkali.

## CHAPTER XXV

### BASAL METABOLISM AND METABOLISM EXPERIMENTS

#### BASAL METABOLISM

Books and General Articles Peters, J P, and Van Slyke, D D, *Quantitative Clinical Chemistry*

Du Bois, E. F, *Basal Metabolism in Health and Disease*

Sanborn, T. B, Editor, *Basal Metabolism Its Determination and Application*

Boothby, W M, and Sandiford I, (a) *Laboratory Manual of the Technic of Basal Metabolic Rate Determinations*, (b) "Basal Metabolism," *Physiol Rev*, 1924, 4, 69

Macleod, J J R, *Physiology and Biochemistry in Modern Medicine*

Beaumont, G E, and Dodds, E C, *Recent Advances in Medicine* (Chapter on "Basal Metabolism")

Talbot, F B, "Basal Metabolism of Children," *Physiol Rev*, 1925, 5, 477

Cathcart, E P, "Discussion on Basal Metabolism," *Brit Med J*, 1922, ii, 747

Douglas C G, and Priestley, J G, *Human Physiology A Practical Course*

CHEMICAL changes are constantly taking place in all the cells and tissues. Some cells die, and the products are removed in part or in whole from the body. New cells are formed to take their place. In other cells a portion of the cell substance is broken down, e g, during muscular activity, and the resulting products are partly or wholly removed in the blood and excreted. The formation of new cells and the repair of the wear and tear of existing living cells is eventually dependent on the intake of suitable foodstuffs which are digested, absorbed and carried to the tissues. All these complicated changes are included in the term "metabolism." The combustion of foodstuffs and the breakdown of tissue products is dependent on oxidation, and therefore the consumption of oxygen may be used as a measure of the metabolism. During this combustion carbon dioxide is formed and eventually excreted, almost entirely by the lungs. The excretion of  $\text{CO}_2$  in the breath may, therefore, also be used as a measure of metabolism. Lastly, these chemical changes are accompanied by the formation of heat, and so the output of heat may be used as a measure of metabolism (calorimetry).

In order to obtain a comparison between the metabolism of healthy and of diseased individuals, it is necessary to make the measurements under standard or "basal" conditions. These

conditions are described below, and are controlled arbitrarily so as to make the measurements with as little discomfort as possible to the subject, and in a practical manner applicable to clinical work. Innumerable experiments have been made to discover the magnitude of the different factors influencing the measurements, and as a result various modifications of the standard conditions have been made by different workers at various times, but those given below now appear to be widely accepted. The results obtained under these conventional conditions are regarded as estimations of the "basal metabolism" ("standard metabolism," Krogh), or of the "basal metabolic rate" (B.M.R.). After studying the conditions agreed upon, it will be obvious that "basal" is not equivalent to "minimum" metabolism. The metabolism during sleep, for instance, is lower than the "basal" metabolism. The same measurements naturally may be utilised in studying the metabolism after exercise, after particular diets, and so on, but, in general, a comparison between health and disease is most readily made under the "basal" conditions.

### CONDITIONS TO BE OBSERVED IN B.M.R. DETERMINATIONS

Preparation of the patient is essential, so that at the time of the test he is at perfect physical and mental rest, and has had no food for twelve to eighteen hours. It is true that abstinence from food for twelve hours or so will influence the metabolism of various individuals slightly differently, depending on the previous diet, etc., but it is the best of the practicable conditions that can be laid down in clinical work, since it only necessitates the loss of breakfast. It has been shown that a light non-stimulating breakfast does not influence the B.M.R. measured two hours later, but in practice it is unwise to permit any such meal, for fear the patient should not adhere strictly to the items allowed.

To avoid muscular activity the test is best made at the bedside. Otherwise the patient may be wheeled on a trolley and kept at rest by the apparatus for half an hour. If observations must be performed on out-patients, instructions must be given that they shall walk as little as possible, and an hour's rest by the apparatus should be enforced. Severe exercise may require several hours of rest before the basal level is regained, so that the preliminary rest period that should be enforced for out-patients who have taken intermediate amounts of exercise cannot be properly defined, and observations on out-patients must be accepted with reserve. During the preliminary rest period all activity (talking, reading, smoking, etc.) is prohibited.

It is more difficult to procure mental than muscular rest, yet mental anxiety and excitement definitely raise the metabolic rate. It is, therefore, necessary to explain to the patient beforehand (*e.g.*, on the previous day) what is going to be done, to show him the apparatus, and to adopt all possible means to insure that he shall be at mental rest before and during the test.

Du Bois has shown that the metabolic rate rises approximately 10 per cent for each  $1^{\circ}\text{C}$  rise of body temperature. The patient's temperature should, therefore, be taken before the test. Moreover, the patient before and during the observation should be comfortably warm, so as to avoid shivering or the increased invisible muscle tension caused by cold. It is questionable whether the conditions in the general wards of most of our hospitals satisfy these criteria.

### METHODS OF DETERMINING THE B M R

The most accurate method is to estimate the quantity of heat given out in a definite time under the standard (basal) conditions, with the aid of a calorimeter, but, in general, its expense is too great for clinical work. The reader is, therefore, referred to larger books for an account of calorimetry and its application.

Alternatively, the oxygen consumption, or  $\text{CO}_2$  excretion, or both are measured. Indeed, the  $\text{CO}_2$  exhaled divided by the  $\text{O}_2$  consumed—the respiratory quotient, or R Q—must be determined if as accurate results as possible are to be secured. Fairly trustworthy figures, however, may be obtained if the R Q is assumed to be a certain value (0.82 is usually adopted), and either the oxygen consumption or the  $\text{CO}_2$  excretion is determined. In this case  $\text{O}_2$  determinations are more satisfactory than  $\text{CO}_2$  estimations, since the calorific value of 1,000 c.c. of  $\text{O}_2$  when fat (R Q, 0.71) is burned is 4.69 calories and when carbohydrate (R Q, 1.00) is burned is 5.05 calories, whereas the corresponding figures for  $\text{CO}_2$  are 6.63 and 5.05 calories respectively. (For explanation of these calculations see Peters and Van Slyke's chapter on Total Metabolism.) Departures from the assumed R Q of 0.82 will, therefore, affect the B M R more if calculated from the  $\text{CO}_2$  values than if calculated from the  $\text{O}_2$  figures.

The gasometric methods are of two main types: 'the closed circuit' and 'the open circuit'. As an example of the former the subject is directly connected with a gasometer filled with oxygen-enriched air, and an absorber for  $\text{CO}_2$ . The circuit is closed so that oxygen + nitrogen is breathed in and oxygen + nitrogen +  $\text{CO}_2$  is breathed out. The  $\text{CO}_2$  being absorbed, the decrease in volume of the oxygen in the gasometer can be directly observed for a measured time.

As an example of an open circuit technique the principle of the Douglas bag method may be given. The patient breathes in air, and expires into the previously emptied bag for a measured time (usually about ten minutes). A sample of the contents of the bag is analysed for  $\text{CO}_2$  and  $\text{O}_2$  with the aid of a Haldane gas analysis apparatus. The volume of gas in the bag is then measured by passing it through a meter.

The closed circuit principle is extensively employed in medicine owing to the simplicity of the appliances recently introduced for clinical work. One of these, "The British 'Benedict' Portable Metabolism Apparatus," is illustrated in Fig. 82, on p. 490. It is

essentially the same as the Benedict-Roth model. In this method gas analysis is not required, but calculation of the B M R, since the oxygen consumption is alone determined, necessitates the assumption that the patient's R Q is 0.82, because the standard oxygen consumption, with which the patient's is compared, is based on an R Q of that value.

Using the Douglas bag method, both the CO<sub>2</sub> and the O<sub>2</sub> are measured, from which is calculated the actual R Q of the patient. Careful gas analysis is required, and the calculations necessary to arrive at the B M R are involved. This technique has been widely employed by laboratory workers, but since it is fully described in the books to which reference has been made at the beginning of this chapter, it will not be further considered here. From the patient's point of view it has been claimed that this open-circuit method is preferable, because he complains less often of subjective symptoms, such as "fullness in the head," headache, and difficulty of breathing, than when he receives oxygen-enriched air as in the closed circuit method. The majority of clinical workers, however, do not find that these symptoms often occur and, when they are complained of, it is only rarely that they are serious enough to interfere with the test.

Rabinowitch and Bazin find that a simple and accurate determination of the B M R may be obtained by allowing the patient to breathe directly into a gasmeter, and obtaining periodically the percentages of CO<sub>2</sub> in the expired air electrometrically by means of a katharometer.

### CLINICAL VALUE

The values for the B M R in health certainly fluctuate between + 10 and - 10 per cent of the standard, and many workers extend the normal range to  $\pm 15$  per cent.

In the great majority of diseases the B M R falls within these normal limits, as shown by the table on p. 487, which is extracted from a longer table published by Boothby and Sandiford.

It is in thyroid diseases that B M R determinations are of most value —

(a) As an aid to diagnosis, particularly in border line cases of hyperthyroidism and hypothyroidism.

(b) As a measure of the severity of obvious thyroid disease.

(c) Repeated at intervals of one or two weeks to indicate progress due to rest in bed and medical treatment.

(d) As an aid to the selection of treatment (general medical, surgical, X rays, radium, or preliminary iodine medication in exophthalmic goitre) and by repetition as a check on the treatment selected.

(e) In the diagnosis between adenoma with and without hyperthyroidism respectively, and in the study of recurrent goitres.

(f) Repeated at intervals of about a month as one of the methods of controlling treatment with thyroxine.

(Boothby and Sandiford, 1924)

*B M R in Conditions not due to Thyroid Diseases*

Diagnosis	No of cases	Percentage range							
		below -20	-20 to -16	-15 to -11	-10 to +10	+11 to +15	+16 to +20	above +20	15 to +15
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Healthy	76	—	—	6	84	9	1	—	99
Migraine	42	—	—	10	83	2	—	—	100
Chronic nervous exhaustion	191	1	1	5	86	6	1	—	9
Neurasthenia	210	—	1	11	80	6	—	"	97
Mental	99	—	4	4	82	7	—	3	91
Epilepsy	24	—	13	8	63	12	—	4	83
Essential hypertension	95	—	3	2	66	11	8	10	9
Cardiac	148	1	1	3	60	19	7	7	84
Renal	80	2	2	14	69	4	1	8	87
Gastro-intestinal	112	1	4	12	3	10	—	—	95
Malignancy	23	13	4	4	48	17	9	5	69
Dermatological	34	—	—	6	74	15	3	2	95
Gynecological	40	5	—	10	83	—	2	—	93
Pregnancy	29	—	—	—	79	10	7	4	89
Obesity	55	2	2	2	80	4	5	5	86
Acromegaly	5	—	—	—	60	—	20	20	60
Hypopituitarism	14	7	15	14	50	—	7	7	64
Addison's disease	12	—	—	17	67	8	8	—	90
Secondary anemia	25	—	—	—	96	—	4	—	96
Anemia aplenic and pernicious	36	3	—	3	53	8	19	14	64
Leukemia lymphatic and myelogenous	15	—	—	—	97	7	6	80	34
Diabetes	61	5	3	5	69	13	2	3	87
Arthritis	65	2	2	6	83	1	6	—	90

Extensive operations for goitre are considered unwise by most surgeons if the B M R exceeds +40 per cent. Boothby and Sandiford's findings in different diseases of the thyroid gland are given on p 488.

The B M R is raised about 2.5 per cent for each mgm of intravenous thyroxine. The maximum effect of thyroxine occurs between the fifth and tenth day after administration. The oral dose required daily by a completely thyroidless adult is of the order of 1.5 mgm, but the preliminary daily dose in myxœdema should not exceed 0.2 mgm. The sodium salt of thyroxine is generally used in therapeutics since it is more soluble than thyroxine itself.



*B.M.R. in Thyroid Diseases*  
(Boothby and Sandford, 1924)

Diagnosis	No of cases	Percentage range									
		above +75	+74 to +50	+49 to +21	+20 to +16	+15 to +11	+10 to -10	-11 to -15	-16 to -20	below -20	above +20
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Exophthalmic goitre	452	12	40	40	4	1	3	—	—	—	92
Recurrent exophthalmic goitre	56	11	16	61	3	2	5	2	—	—	88
Adenoma with hyperthyroidism	346	2	11	52	13	22	—	—	—	—	65
Adenoma without hyperthyroidism	544	—	—	—	—	—	100	—	—	—	—
Colloid goitre . . . .	140	—	—	1	1	7	81	4	5	1	1
Myxœdema . . . . .	12	—	—	—	—	—	—	17	—	83	—
Post operative myxœdema	11	—	—	—	—	—	—	9	9	82	—
Questionable hypothyroidism	42	—	—	—	—	—	26	19	24	31	—
Thyroiditis . . . . .	27	—	4	41	—	7	44	4	—	—	45
Malignant thyroid . . .	24	—	8	4	4	13	58	—	13	—	12

For some unknown reason the B M R is often raised in leukaemia (*cf* table on p 487) Determinations in pituitary disease are of less value than in thyroid disease In thirty cases of acromegaly, Boothby and Sandiford (1922) found eighteen to have B M R's within normal limits ( $\pm 15$  per cent), one below normal, and eleven above normal (in eight the B M R exceeded  $+ 20$  per cent) In fifty-eight cases of hypopituitarism their results were as follows — B M R  $\pm 15$  per cent in thirty-two, above  $+ 15$  per cent in four, and below  $- 15$  per cent in twenty two patients In only about a third of these cases, therefore, was there a definite depression of metabolism The interpretation of the results in diabetes mellitus is a matter of controversy It is agreed, however, that in the majority the B M R is within normal limits, and that of the rest some have a raised and others a lowered B M R In obesity the determinations are occasionally of value as part of the chain of evidence pointing to an endocrine factor, but in the great majority of obese subjects the B M R is within normal limits (*cf* table on p 487) Porter found a B M R of below  $- 20$  per cent in six out of ten children, and in two out of eight adults with ichthyosis In chronic parenchymatous nephritis (nephrosis) the B M R is often subnormal and it has been claimed that thyroid administration is of value Some workers however, attribute the low B M R to the inclusion of metabolically inactive oedema fluid in the body weight upon which the B M R is calculated In other renal diseases the B M R is not low

The basal metabolism of children is reviewed by Talbot He maintains that a respiratory chamber is necessary for children under eight years The "basal" metabolism of infants under two years is not strictly basal because it is measured with food in the stomach This makes the observed B M R too high by  $+ 8$  to  $+ 15$  per cent He considers that it is most satisfactory to determine the B M R in reference to height for both boys and girls, from the age of two weeks to twelve years He reviews the results that have been obtained in different diseases in children, and notes the unfrequency of such observations In untreated cretinism the B M R is always low The difficulty is to secure measurements before thyroid medication has been started The effect of such treatment on the B M R may not wear off for three or four weeks, or even longer

With regard to drugs, with the exception of thyroxine, of adrenaline, and of iodine in exophthalmic goitre and in some cases of adenoma of the thyroid, it may be stated that none affects the B M R significantly

#### TECHNICAL

The technique of B M R determinations with the aid of the British "Benedict" Portable Metabolism Apparatus<sup>1</sup> will be

<sup>1</sup> Obtainable from Messrs H E Kendrick, 5 Lodge Villas Woodford New Road, Woodford Green Essex

described, since it is the only type of method likely to be used by the practitioner.

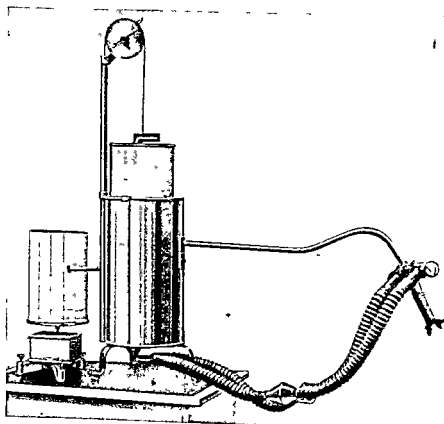


FIG 82. The British "Benedict" Portable Metabolism Apparatus.

The apparatus is illustrated above. The patient is connected with the spirometer by means of a mouthpiece and breathing tubes fitted with inlet and outlet valves, breathing through the nose being prevented by a nose-clip. The spirometer is a small gasometer with a water seal. The floating bell is suitably counterpoised, and a revolving dial is attached to the pulley over which the string connecting the bell and counter-weight runs. This dial is calibrated to record the change in volume of the gas in the spirometer. Soda-lime (e.g., "Calsoda," or "Carbosorb self-indicating granules (mesh 4 to 10) B.D.H.") is placed inside the spirometer to absorb  $\text{CO}_2$ .

Oxygen is introduced into the spirometer. The mouthpiece (disconnected) is placed in the patient's mouth, and his nose is clipped. When the patient is accustomed to breathing under these conditions, the mouthpiece is connected with the apparatus. When the operator is satisfied that the patient's breathing is natural,

he takes the time, and notes the volume on the dial. Two observations of four to seven minutes each are made, and the total volume of oxygen consumed and the sum of the different periods are noted. The c.c. of oxygen consumed per minute are then calculated, and reduced to standard temperature and pressure. Knowing the patient's height, weight, age and sex, the B.M.R. is calculated as shown in the following example, with the aid of the tables at the end of this section —

**Data** Male, aged seventeen, weight 139 lb., and height 69 in

Volume of oxygen consumed per minute was 250 c.c. Temperature 18° C and barometer reading 750 mm

**Correction of O<sub>2</sub> to S.T.P.** From p. 493 the aqueous vapour pressure at 18° C is 15.4 mm

From p. 493 the correction for the expansion of the brass scale<sup>1</sup> of the barometer is 2.15 mm

Therefore, the correct pressure is  $750 - 15.4 - 2.15 = 732.45$  mm

To reduce to 0° C and 760 mm, the factor corresponding to 18° C and 732.45 mm is read off from p. 494. It will be found to be 0.904

The corrected volume of oxygen is therefore  $250 \times 0.904 = 226$  c.c.

**Alternative Method of Correction** Many workers will find the calculation more easily made with the aid of the usual formula —

$$V = v \times \frac{p - a}{760} \times \frac{273}{273 + t},$$

where  $V$  is the corrected and  $v$  the observed volume of oxygen,  $p$  is the barometric pressure,  $a$  the aqueous vapour pressure, and  $t$  the temperature. Neglect of the expansion of the brass scale of the barometer introduces very little error, but the expansion factor is easily introduced into the above formula if desired.

In the above example,

$$\begin{aligned} V &= 250 \times \frac{750 - 15.4}{760} \times \frac{273}{291} \\ &= 250 \times \frac{734.6}{760} \times \frac{273}{291} = 227 \text{ c.c.} \end{aligned}$$

**Calculation of B.M.R.** The next step is to compare the patient's oxygen consumption with that of a normal person of the same height, weight, sex and age. By reference to p. 495 it is seen that the standard oxygen consumption of a subject weighing 139 lb.,

<sup>1</sup> A brass scale is seldom fitted nowadays

and with a height of 69 in, is 228 c c per minute This must be corrected for age and sex as follows —

Males	Aged 20 to 50, no correction
"	16 to 18, add 10 per cent to volume in table
"	18 to 20, add 5 per cent " "
"	50 to 60, subtract 4 per cent from volume in table
"	60 to 70 subtract 7 per cent " "

Females Calculate as for male of same age, and subtract 7 per cent

In our example (male, aged seventeen) 10 per cent of 228 c c must be added, making the correct standard oxygen consumption  $228 + 22.8$  c c, or 251 c c

The standard oxygen consumption is 251 c c, the patient's is 226 c c or 25 c c below the standard

The B M R is expressed as a percentage increase or decrease on the standard corrected volume Therefore, the patient's B M R is  $-\frac{25}{251} \times 100$  or - 10 per cent

Notes The figures for the standard oxygen consumptions on pp 495-496 have been calculated on the assumption that the R Q is 0.82 Reference to this point has been made above The B M R is computed from the area of the body surface The figures on pp 495-496 have been calculated with the aid of Du Bois' formula for surface area

The technique is simple, but to obtain satisfactory results the following additional points must be observed —

- (1) The entire apparatus must be thoroughly tested for leaks
- (2) The spirometer cylinder must be accurately balanced by means of the four levelling screws and the pulley counter weight
- (3) The CO<sub>2</sub> absorber must be systematically examined, e.g., once or twice a week The capacity of the soda lime to absorb CO<sub>2</sub> is tested by filling the spirometer bell with expired air, plugging the mouthpiece and causing the contents to pass out *via* the oxygen inlet tap and bubble through lime water If the soda lime is all right no cloudiness will occur Alternatively cal soda is renewed after every thirty tests or after some other total which has been shown by experiment (as above) to yield a safe margin, the indicator in "carborsorb" gives the warning Of course the lid of the soda lime container must be removed before a test

(4) It is essential that the readings on the dial at the beginning and end of a test shall be taken in the course of periods of steady regular fall in the volume, as shown by the hand on the dial, or by the respiratory tracing when the means for securing the same are supplied (see Fig 82 on p 490) A stop watch is of great service

It is an excellent plan to take readings every minute, by which means alterations in breathing or a sudden leak, e.g., past the nose clip, is promptly detected The following is an example of a satisfactory experiment —

Time Minutes	Dial Reading	C c O <sub>2</sub> per min	Time Minutes	Dial Reading	C c O <sub>2</sub> per min
0	5,800	—	0	5,560	—
1	5,460	340	1	5,240	320
2	5,100	360	2	4,880	360
3	4,780	320	3	4,540	340
4	4,480	300	4	4,280	260
5	4,200	280			
6	3,860	340			
7	3,560	300			

O<sub>2</sub> per min for first period = 320 c c and for second period = 320 c c

(5) Other points, which though very elementary have been known to be the cause of errors, are as follows —

(a) The oxygen inlet tap must be turned off after filling the gasometer.

(b) If too much water is put in the water seal it will spill over with each inspiration as the bell falls, and spoil the soda-lime

(c) The temperature of the experiment is of course that recorded by the thermometer on the top of the bell

### TABLES FOR CALCULATION OF B.M.R.

#### Tension of Aqueous Vapour in Millimetres of Mercury

To obtain the dry barometer pressure, subtract the mm Hg corresponding to the temperature of the air from the barometer pressure at the time of the experiment

Temp.	15°	16°	17°	18°	19°	20°	21°	22°	23°	24°	25° C
Mm	12.7	13.5	14.4	15.4	16.3	17.4	18.5	19.7	20.9	22.2	23.5

(Macleod, *Physiology and Biochemistry in Modern Medicine*)

#### Temperature Corrections to Reduce Readings of a Mercurial Barometer with a Brass Scale to 0° C.

Subtract the appropriate quantity as found in the table from the height of the barometer. The table is for a barometer with a brass scale, and the values are a little lower (about 0.2 mm.) than for the glass scale. The corrections for intermediate temperatures can be approximated by interpolation

Temp	700 mm	710 mm	720 mm	730 mm	740 mm	750 mm	760 mm	770 mm
15°	1.69	1.72	1.74	1.77	1.79	1.81	1.84	1.86
20°	2.26	2.29	2.32	2.36	2.39	2.42	2.45	2.48
25°	2.83	2.87	2.91	2.95	2.99	3.03	3.07	3.11

(Macleod, *Physiology and Biochemistry in Modern Medicine*)

### Table for Reducing Gaseous Volumes to Normal Temperature and Pressure

The observed volume, when multiplied by the factor corresponding to the temperature and corrected pressure, will give the volume of the expired air reduced to 0° and 760 mm

Mm	15°	16°	17°	18°	19°	20°	21°	2°	23°	24°	25°
720	898	894	891	888	885	882	880	877	873	870	867
730	910	907	904	901	897	894	891	888	885	882	879
740	922	919	916	913	910	907	904	901	897	894	891
750	935	932	928	925	922	919	916	913	910	907	904
760	947	944	941	938	934	931	928	925	922	919	916
770	960	957	953	950	948	945	940	936	933	930	927

(Macleod *Physiology and Biochemistry in Modern Medicine*)

Standard O<sub>2</sub> Consumption Relative to Height and Weight

		HEIGHT IN CENTIMETERS															
		140	145	150	155	160	165	170	175	180	185	190					
30	1	142	145	149	152	156	159	163	166	170	173	177	66				
1	2	144	147	151	154	158	161	165	168	172	175	179	68				
2	3	145	149	153	156	160	163	167	170	174	177	181	70				
3	4	147	151	155	158	162	165	169	173	177	180	184	73				
4	5	149	153	157	160	164	167	171	175	179	182	186	75				
5	6	151	155	159	162	166	170	174	177	181	184	188	77				
6	7	153	157	161	164	168	172	176	179	183	187	191	79				
7	8	155	159	163	166	170	174	178	182	185	189	193	81				
8	9	156	161	164	168	172	176	180	184	188	191	195	84				
9	1	158	162	166	170	174	178	182	186	190	194	198	86				
40	1	159	164	168	172	176	180	184	188	192	196	200	88				
1	2	161	165	169	174	179	182	186	190	194	198	202	90				
2	3	162	167	171	176	181	184	188	192	196	200	204	92				
3	4	164	169	173	178	182	185	189	194	198	202	206	94				
4	5	165	170	175	180	184	187	191	196	200	204	208	97				
5	6	167	172	177	181	186	189	193	198	201	205	210	99				
6	7	169	174	179	183	188	191	195	200	203	207	212	101				
7	8	171	176	180	184	189	192	197	202	205	209	214	103				
8	9	172	177	182	186	191	194	199	204	207	211	216	105				
9	1	174	179	183	188	192	196	201	206	209	213	218	108				
50	1	176	181	185	189	193	197	202	207	211	215	220	110				
1	2	178	183	186	191	195	199	204	209	212	217	221	112				
2	3	179	184	188	192	196	200	205	211	214	219	223	114				
3	4	181	186	189	194	198	202	207	212	216	220	225	117				
4	5	182	187	191	195	199	203	208	214	217	222	227	119				
5	6	184	188	193	197	201	205	210	215	219	224	228	121				
6	7	185	189	194	199	203	207	212	218	221	226	230	123				
7	8	186	191	196	200	205	209	213	219	222	228	232	125				
8	9	188	192	197	202	206	210	215	221	224	229	233	128				
9	1	189	194	199	203	208	212	216	222	225	231	235	130				
60	1	190	195	200	204	208	213	217	223	226	232	237	132				
1	2	191	196	201	206	211	215	219	225	230	235	239	134				
2	3	192	198	202	207	212	216	220	226	231	236	240	136				
3	4	193	199	203	209	214	218	222	228	233	238	242	139				
4	5	195	200	205	210	215	219	223	229	234	239	243	141				
5	6	196	201	206	212	217	221	225	231	236	241	245	143				
6	7	197	202	207	213	218	222	227	232	237	242	247	145				
7	8	199	204	209	215	220	224	228	234	239	244	249	147				
8	9	200	205	210	216	221	225	230	235	240	245	250	150				
9	1	201	207	212	218	222	227	231	237	242	247	252	152				
70	1	202	208	213	218	223	228	233	238	243	248	253	154				
1	2	203	209	214	219	224	229	235	240	245	250	255	156				
2	3	204	210	215	220	225	231	236	241	246	251	256	158				
3	4	206	212	217	222	227	232	238	243	248	253	258	161				
4	5	207	213	219	223	228	234	239	244	249	254	259	163				
5	6	208	214	220	224	229	235	240	245	251	256	261	165				
6	7	209	215	221	225	230	236	242	247	252	257	263	167				
7	8	210	216	222	227	232	238	244	249	254	259	264	169				
8	9	212	218	224	229	233	239	245	250	255	260	266	172				
9	1	213	219	225	230	235	240	246	251	256	262	267	174				
80	1	214	220	226	231	236	241	247	253	258	263	268	176				
1	2	215	222	227	232	237	242	248	254	259	264	270	178				
2	3	217	223	228	234	239	244	250	256	261	266	271	180				
3	4	218	225	230	235	240	245	251	257	262	267	273	183				
4	5	220	226	231	237	242	247	253	259	264	269	274	185				
5	6	221	227	233	238	243	248	254	260	265	270	276	187				
6	7	222	228	234	239	244	249	255	261	266	271	277	189				
7	8	223	229	235	240	246	251	256	262	268	273	279	191				
8	9	224	230	236	242	247	252	258	264	269	274	280	194				
9	1	225	231	237	243	248	254	259	265	271	276	282	196				
		53	57	59	61	63	65	67	69	71	73	75					
		HEIGHT IN INCHES															



Standard O<sub>2</sub> Consumption (continued)

		HEIGHT IN CENTIMETERS																	
		150	155	160	165	170	175	180	185	190	195	200							
90	238	244	249	255	260	267	271	278	282	287	292	297	198						
1	239	245	250	256	261	267	272	277	283	288	293	298	200						
2	240	246	251	257	262	268	273	278	284	289	294	299	202						
3	241	247	252	258	264	269	275	280	286	291	296	301	205						
4	242	248	253	259	265	270	276	281	287	292	297	302	207						
5	243	249	254	260	266	271	277	282	288	293	298	303	209						
6	244	250	255	261	267	272	278	283	289	294	299	304	211						
7	245	251	256	262	268	274	279	284	290	295	300	305	213						
8	246	252	258	264	270	275	281	286	292	297	302	307	216						
9	247	253	259	265	271	277	282	287	293	298	303	308	218						
100	248	254	260	267	272	278	284	289	294	299	304	309	220						
1	249	255	261	268	273	279	285	290	295	300	305	310	222						
2	250	256	262	269	274	280	286	292	297	302	307	312	224						
3	251	257	263	270	275	281	288	293	298	303	308	313	227						
4	252	258	264	271	276	282	289	294	299	304	309	314	229						
5	253	260	266	272	278	284	290	296	301	306	311	316	231						
6	254	261	267	273	279	285	291	297	302	307	312	317	233						
7	255	262	268	274	280	286	292	298	303	308	313	318	235						
8	256	263	269	275	281	287	293	299	305	310	315	320	238						
9	257	264	270	276	282	288	294	300	306	311	316	321	240						
110	258	265	271	277	283	289	295	302	307	312	317	322	242						
1	259	266	272	278	284	290	297	303	308	313	318	323	244						
2	260	267	273	279	285	291	298	304	309	314	319	324	246						
3	261	268	274	280	286	292	299	305	310	315	320	325	249						
4	262	269	275	281	287	294	300	306	311	317	322	327	251						
5	263	270	276	282	288	295	302	308	313	318	323	328	253						
6	264	271	277	284	290	296	303	309	314	320	325	330	255						
7	265	272	278	285	291	298	304	310	315	321	326	331	257						
8	266	273	279	286	292	299	305	311	317	322	328	333	259						
9	267	274	280	287	293	300	306	312	318	324	329	334	262						
120	268	275	281	288	294	301	307	313	319	325	330	336	264						
1	269	276	282	289	295	302	308	314	320	326	331	337	266						
2	270	277	283	290	296	303	309	315	321	327	332	338	268						
3	271	278	284	291	297	304	310	316	322	328	333	339	271						
4	272	279	285	292	298	305	311	317	323	329	335	341	273						
5	273	280	286	293	299	306	312	318	324	330	336	342	275						
6	273	280	286	293	300	307	313	319	325	331	337	343	277						
7	274	281	287	294	301	308	314	320	326	332	338	344	279						
8	275	282	288	295	302	309	315	321	327	333	339	345	282						
9	276	283	289	296	303	310	316	322	328	334	340	346	284						
130	277	284	290	297	304	311	317	323	329	335	341	347	286						
1	278	285	291	298	305	312	318	324	330	336	342	348	288						
2	279	286	292	299	306	313	319	325	331	337	343	349	290						
3	280	287	293	300	307	314	320	327	333	339	345	351	293						
4	281	288	294	301	308	315	321	328	334	340	346	352	295						
5	281	288	294	301	308	315	321	328	334	340	346	352	297						
6	282	289	295	302	309	316	322	329	335	342	348	354	299						
7	283	290	296	303	310	317	323	330	336	343	349	355	301						
8	284	291	297	304	311	318	324	331	337	344	350	356	304						
9	285	292	298	305	312	319	325	332	338	344	350	356	306						
140	286	293	299	307	313	320	327	333	339	345	351	357	308						
1	287	294	300	308	315	322	329	335	341	347	353	359	310						
2	288	295	301	309	316	323	329	336	342	348	354	361	312						
3	289	296	302	310	317	324	330	337	343	349	355	362	315						
4	289	296	303	311	317	325	331	338	344	350	356	363	317						
5	290	297	304	312	318	326	332	339	345	351	357	364	319						
6	291	298	305	313	319	327	333	339	346	352	358	365	321						
7	291	299	306	314	320	328	334	340	346	353	359	366	323						
8	292	300	307	315	321	329	335	341	347	354	360	367	326						
9	293	301	308	316	322	330	336	342	348	355	361	368	328						
150	294	302	309	317	323	331	337	343	349	355	361	368	330						

HEIGHT IN INCHES  
(From Sanborn's Basal Metabolism)

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## METABOLISM OR "BALANCE" EXPERIMENTS

**Books** Lusk, G, *The Science of Nutrition*

Hawk P B, and Bergeim, O, *Practical Physiological Chemistry*, chapter on Studies in Metabolism

Sherman, H C, *Chemistry of Food and Nutrition*

McLester, J S *Nutrition and Diet in Health and Disease*

Several references have been made in previous chapters to the necessity for making metabolism observations before the significance of certain analyses can be assessed. Thus it is obviously useless to estimate the output of a substance in the urine if that output is largely dependent on the intake of the substance or its precursor in the diet, unless the intake is also measured. For example the output of urea depends mainly on the protein intake. Again if the substance is excreted by both the kidneys and the bowel observations limited to the urine are incomplete, and so on.

In clinical work metabolism investigations can rarely be undertaken satisfactorily except in institutions with a specially trained nursing staff. Moreover, the time and expense involved practically limit such observations as are made in general hospitals to the investigation of a few special problems and to research work. For these reasons only a very brief outline of metabolism experiments will be given with sufficient information to enable the reader to understand the general principles, and to realise why so many analyses are of very limited value unless they form part of a proper metabolism investigation.

### GENERAL CONSIDERATIONS

In planning metabolism experiments it is obviously necessary to know by what paths the substance under investigation is excreted. In accurate work it is necessary to analyse the food to determine the intake of the particular ingredient. In clinical work, however, sufficient information may frequently be obtained by weighing the articles of diet, and computing the intake of the substance with the aid of tables recording the average analyses of foods. Alternatively certain problems may suitably be studied by placing the patient on a "constant diet" for a few days before and during the test period. In that case it is obviously necessary to weigh each article of food, and to insure that the same quantities of the same items are ingested each day. Usually there should be a preliminary period of two or three days on the special diet, followed immediately by the test period of three or more days, depending on the nature of the investigation. It cannot be too strongly stressed that rapid changes in diet (e.g., every third day) and short

test periods (*e g*, one or two days) often not only give no useful information, but may actually be misleading

When the results of metabolism investigations are studied, it may be evident that the quantity of the substance excreted is less than the amount ingested, *i e*, some has been retained (or destroyed) in the body, in other words, there is a "positive balance" Conversely, the results may indicate a "negative balance" or, in other words, some of the substance excreted must have been derived from the body, and not from the food In continuation of the same phraseology, metabolism observations are often referred to as "balance experiments"

It is seldom difficult to measure the intake and output of a given substance The difficulty is to know how to interpret the results For example, how far is it justifiable to assume that when  $x$  gm of a substance are ingested, and 90 per cent is excreted, that the whole of the 90 per cent recovery represents material that was ingested? May, for instance, 20 per cent of the substance be stored or be utilised in the body, and 10 per cent be simultaneously liberated from the body? Obviously the measurement of intake and excretion can give only the algebraic sum of changes which may be simple or may be very complex Further insight into the processes taking place intermediately may be obtained by studying the excretion when the intake of  $x$  is *nil*, either by giving a diet free from  $x$  (or its precursors), or by giving no diet at all (*i e*, by studying the "fasting metabolism") Again, further information may result from blood analysis, and so on

The collection and preservation of blood and of excreta have been previously described In adults there is seldom much difficulty in obtaining the requisite excreta for analysis, though often greater difficulty is experienced with female than with male patients, owing to the problem of collecting separately the urine and the feces, and the occurrence of menstruation In infants and young children the practical obstacles are greater, for which reason various mechanical devices have been introduced, *e g*, the "Hoobler" metabolism bed On the other hand, the fact that an infant's diet generally consists of the single article, milk, is an advantage

The observations that may be made include the metabolism of carbohydrates, proteins and other nitrogenous substances, fats, salts, and water, and the study of the acid base balance and of vitamins The last is outside the scope of this book The acid base balance has been dealt with in Chapter IX A brief account of the clinical application of the other metabolic studies follows

### WATER METABOLISM

Water constitutes 60 to 70 per cent of the body weight It is derived from three main sources, the water consumed as such and in other liquids, the water in so called solid foods which is quite as important as the water in liquid foods, and the water formed during the oxidation of carbohydrates, proteins and fats Some animals, *e g*, rabbits and guinea pigs, can obtain all the water

they require from "solid" foods, and some human beings excrete in the urine more water than is contained in the liquid part of their nourishment. It is clear, therefore, that the clinical practice of measuring the fluid intake, and comparing it with the volume of urine excreted per diem, is of doubtful value, since the water ingested in solid food and the water lost in the faeces, in the breath and through the skin, is not measured.

As a rule a little over half the total water elimination occurs in the kidneys,<sup>1</sup> but in certain diseases this proportion may be greatly reduced without serious results. It is well recognised that enormous losses of water may occur in the faeces in severe diarrhoea, but the smaller but still considerable loss which may occur in stools which are not grossly liquid is probably not sufficiently appreciated. Loss of water by vomiting, in discharges, etc., has also to be considered in disease.

It has been shown by Benedict and by others that under ordinary conditions 250 to 400 gm of water may be lost daily in the expired air. An increased respiration rate, fever, a lowered air temperature, and a lower humidity all increase this loss.

Soderstrom and Du Bois have shown that on the average 700 gm of water daily are lost through the skin and lungs without any sensible perspiration, both in health and in disease, including patients with oedema. With sweating the loss is greater. Du Bois, in his interesting chapter, of which the above is an extract, suggests that as a rough clinical test, water intake may be represented by the total daily weight of all liquid and solid foods, and water output as the day's total weight of urine, faeces (sputum, vomit, etc.), plus 700 gm to represent water vaporised from skin and lungs (Allow 600 gm for a small person, 800 gm for a large person, at rest).

Incidentally a consideration of the above figures shows how absurd it is to attach any clinical significance to small gains or losses in body weight unless such small plus or minus variations are progressively up or down over a prolonged period of observation.

The practice of gauging the degree of oedema and/or invisible water retention by weighing the patient (daily) is sound. Du Bois states "It is difficult to say how great the accumulation of extra fluid in the body must be before it is detected by the eye. In some cases there may be, perhaps, a gain of 2 kilograms without visible oedema, in others, a fraction of this amount may become apparent in a puffiness of the eyelids or slight pitting of the shins."

The admitted difficulties in making accurate balance experiments for water, even under the carefully controlled conditions of scientific investigations, is sufficient "excuse" for the clinician's neglect of water metabolism. There is, however, no excuse for neglecting scientific observations which are quite accurate enough to demonstrate that some of the past cherished clinical beliefs were little short of ridiculous.

<sup>1</sup> Thus in health at rest and comfortably warm, approximate quantities are, in urine 1 500 c c., in faeces, 100 c c., loss by skin and lungs, 700 c c. daily.

A brief account of hydraemia and anhydraemia is given in Chapter XIX under "Volume of Whole Blood and Plasma" A fuller description will be found in Chapter VIII of de Wesselow's *Chemistry of the Blood in Clinical Medicine* Rowntree gives an excellent review of the water balance of the body, including a brief account of "water intoxication" due to excessive intake of water

### CARBOHYDRATE METABOLISM

In clinical work studies of carbohydrate metabolism are practically limited to diabetes mellitus, and to the various "glycosurias" which have already been fairly fully considered in Chapters VII and VIII

The intake is generally computed with the aid of tables based on average analytical findings This procedure is commonly accurate enough for the purpose, but the reader is referred to the work of McCance and Lawrence, which indicates that the analyses of many cooked vegetables and of some fruits require revision, and that only the "available" carbohydrate should be considered when calculating the carbohydrate intake with the aid of tables Analyses of output are almost always confined to the urine, since the faecal carbohydrate is small and is probably largely derived from the bacterial decomposition of celluloses, etc In the writer's opinion the necessity of careful supervision of the collection of the twenty four hours urine cannot be stressed too strongly, satisfactory collection is difficult, if not practically impossible, in the general wards of a hospital This does not imply that the nursing staff is inefficient It is almost inevitable under the conditions of a busy general ward in which several nurses, some of whom are still in training, are in turn responsible for the collection (and the diet) This statement is based on the observation that under the ordinary ward conditions with a diabetic patient on a fixed intake of carbohydrate, protein fat and calories for several weeks (without insulin) the output of glucose fluctuated about  $\pm 20$  gm, whereas when the same patient collected the urine himself the fluctuation did not exceed  $\pm 5$  gm daily

In the writer's opinion another very important point is frequently overlooked, viz, the necessity of making the test periods sufficiently long The effect of a change in diet may last for several days, and each change should be followed by urinary analyses for at least six or seven days before another change is made The rapid changes (eg, with two-day intervals) in diet, used in certain systems of treatment, are, of course, essential if those forms of treatment are to be carried out in a reasonable time The point is that the measurements of carbohydrate intake and of glucose excretion, when such quick changes are made, should not be regarded as constituting proper balance experiments

There is room for further observation on the carbohydrate content of normal urine, and of non diabetic urines, under the

conditions of careful metabolic investigations. Work on these lines has been reported by Page and others.

Blood sugar curves and their interpretation are discussed in Chapter VII.

## PROTEIN AND NITROGEN METABOLISM

References to the excretion of nitrogenous bodies in the urine have been made in Chapter XVI, and the effect of varying the protein intake is illustrated in the table on p. 295. Nitrogen in the faeces is discussed in Chapter XXIV. For accurate work it is necessary to determine the nitrogen ingested in the food, and the nitrogen excreted in both urine and faeces, the results of such an experiment are illustrated on p. 505. Since, however, the faecal nitrogen forms but a small proportion (generally 5 to 10 per cent) of the total nitrogen excreted in urine plus faeces, its estimation is frequently omitted in clinical work, or an allowance of 1 gm. of faecal nitrogen daily is made (see p. 472). Moreover, the nitrogen intake is often calculated from tables showing the average nitrogen content of foods. Admittedly such a procedure cannot give more than an approximate measure, but it suffices to show any gross positive or negative balance. In clinical work it is rarely necessary to undertake such tests, but it is very important for the clinician to realise the futility of estimating the urinary excretion per diem of total nitrogen, urea, uric acid, etc., without a knowledge of the intake. To express the position in other words, the measurement of the day's output in the urine of total nitrogen or of urea gives an approximate indication of the quantity of protein the patient is ingesting.

The excretion of nitrogen during fasting is greater than during a nitrogen free diet containing carbohydrate and/or fat. Likewise the nitrogen excretion on a diet largely limited to protein is greater than the nitrogen excretion on the same diet plus added carbohydrate and/or fat. In other words, carbohydrate and fat are said to "spare" protein, because when the energy requirements of the body are supplied by these foodstuffs, less of the body protein has to be broken down for energy purposes. Indeed, if sufficient carbohydrate and/or fat are supplied in a nitrogen free diet, the nitrogen excretion represents solely the endogenous nitrogen metabolism, i.e., nitrogen liberated by the death and the wear and tear of the cells in the body. For further information the reader is referred to the physiological text books. From the clinical point of view it is evident that nitrogen excretion not only depends on the intake of nitrogen, but also on the sparing action of the carbohydrates and fats simultaneously ingested in a mixed diet. It is also clear that, in selecting a "constant" diet for clinical work, it is necessary for the selected diet to be sufficient for caloric requirements, if the addition of nitrogenous foods, or the action of certain drugs, and so on, is subsequently to be studied.

The above remarks apply with equal force to studies of sulphur metabolism (cf table on p 289)

## FAT METABOLISM

Fats are excreted entirely in the faeces. A discussion of faecal fat will be found in Chapter XXIV, including an explanation of the use of the term "fat" to include all material soluble in ether or other fat solvents. In metabolism experiments it is, therefore, only necessary to estimate the fat in the food and the fat excreted in the faeces. In clinical work the fat in the diet is generally calculated with the aid of tables giving the average percentages.

It should be remembered that some of the fat in the faeces may have been excreted into the bowel from the blood, probably via the bile. In other words, the faecal fat is the sum of that unabsorbed plus that excreted into the bowel. The faeces during fasting contain small quantities of fat, which probably represent that excreted, together with that derived from desquamated epithelial cells and from bacteria.

Fat metabolism observations are most often made in children to study the retention in intestinal disease, in congenital steatorrhoea, etc. They may also be requested in investigating hepatic and pancreatic disorders in both children and adults. The metabolism period should be three days at least. More accurate results are obtained with longer periods—up to seven days. A metabolism bed is necessary for infants and young children.

Tidwell and Holt have shown that in normal infants at least 90 per cent of the so called "neutral fat" consists of unsaponifiable matter. In certain pathological conditions, or with diets containing poorly absorbed fats, the true neutral fat (triglycerides) does rise, but the only way of showing this satisfactorily is to determine the daily output of each of the fat fractions—their percentage distribution may be misleading.

The following table, from a paper by Dr Sheldon and the writer, gives the fat retention findings of Holt, Courtney and Fales for normal and abnormal children, and our own results (four day metabolism periods) in two cases of coeliac disease.

In normal infants who are breast fed, over 90 per cent of the ingested fat is retained. On modifications of cow's milk, fat retention is not quite so good, but in most cases it exceeds 70 per cent. In older children on mixed diets it generally exceeds 90 per cent.

In rickets fat retention is usually normal. Simple diarrhoea often reduces the quantity of fat retained, in some instances considerably. In chronic intestinal indigestion there is frequently a considerable loss of fat in the faeces. In fact, so far as analytical data are concerned, there is no difference between typical coeliac cases and some of the more severe examples of chronic intestinal indigestion.

## Balance Experiments for Fat

Condition.	Age		Diet.	Fat Intake, gm daily		Fat Output, gm daily		Fat Retention, per cent			No of Observa- tions
	Max	Min		Max	Min	Max	Min	Max	Min.	Av.	
Normal . . .	10 mths	3 weeks	Breast milk.	42.5	9.8	2.28	0.27	99.2	90.3	95.1	8
Diarrhoea . . .	5½ months.		Breast milk		7.0		1.48	—	—	79.0	1
Ileocolitis . . .	8 months		Breast milk .		15.7		5.71	—	—	92.0	1
Acute diarrhoea . .	6 months		Breast milk .		25.2		12.00	—	—	52.5	1
Normal . . .	18 mths.	2 mths	Modifications of cow's milk.	48.3	11.4	7.57	0.38	97.4	71.9	88.6	67
Diarrhoea . . .	14 mths	2½ mths.	Modifications of cow's milk.	29.7	11.6	14.54	1.77	19.1	12.9	74.3	29
Normal . . .	10 years	1 year	Mixed. . .	61.0	21.8	5.65	0.71	98.0	86.3	93.7	78
Rickets . . .	4 years 4 mths	1 year 5 mths	Mixed. . .	61.0	19.6	8.59	1.49	97.3	78.4	91.6	16
Intestinal indigestion .	9 years 4 mths	2 years 1 mth	Mixed . .	74.2	12.7	18.67	1.95	91.1	26.5	78.4	21
Cœliac . . .	21 months.		Mixed . .		10.33 <sup>1</sup>		6.63	—	—	35.8	1
Cœliac . . .	3 years		Mixed. . .		13.06 <sup>1</sup>		2.91	—	—	77.5	1

<sup>1</sup> Note that the intake of fat is low



## SALT METABOLISM

Metabolism studies may be made with regard to sodium, potassium, chlorine, calcium, phosphorus, magnesium, or iron

The urinary excretion of chlorine has been referred to in Chapter XV. The faeces contain practically no chlorides

Salts of calcium, magnesium and iron are not readily absorbed. Moreover, they are all said to be excreted into the large intestine. Usually about 80 to 90 per cent of ingested calcium, and about 60 to 70 per cent of magnesium, are eliminated in the faeces. The ratio of faecal to urinary phosphorus varies considerably, but the proportion in the faeces is commonly 20 to 50 per cent. A higher proportion of calcium tends to be eliminated in stools containing an excess of fatty acids.

The urinary excretion of these salts has been mentioned in Chapter XV (tables, pp. 289 and 294). In metabolism investigations the quantities should be determined in the food as well as in the urine and faeces. The clinical expedient of computing the intake with the aid of tables is of doubtful utility, except when gross abnormalities are revealed, because the composition recorded is the average of analyses showing variations of the order of  $\pm 10$  to 20 per cent of the mean (*cf.* McCance and Shipp).

In clinical work balance experiments are undertaken most often for calcium and phosphorus in rickets and other bone diseases, in tetany and lately in generalised osteitis fibrosa (hyperparathyroidism). A good and simple account of the complex nature of calcium metabolism is given by Herbert. It is essential to realise that in all conditions including health, a negative calcium balance will be obtained if the intake is limited to 100 or 200 mgm of Ca daily; the calcium in the diet must be 400 to 500 mgm daily before absorption can keep pace with excretion. These balance experiments are laborious and time consuming and must of necessity be confined to special investigations. Telfer has reported an interesting series of observations on calcium and phosphorus metabolism. Mineral metabolism in relation to acid base equilibrium is reviewed by Shohl. Sherman estimated that an average intake of 0.45 gm of calcium (as Ca) and 0.88 gm of phosphorus (as P) per 70 kgm of body weight, are required daily for maintenance.

In clinical work it is important to realise that estimations of urinary calcium and phosphorus are practically valueless except as part of a metabolism test.

The following table illustrates the results of a balance experiment.

(From Hawk and Bergeim's *Practical Physiological Chemistry*)

*Balance of Calcium Magnesium Phosphorus Sulphur and  
Nitrogen in Acromegaly (partly recalculated)*

	Calcium	Magnesium	Phosphorus	Sulphur	Nitrogen
	Grams				
Ingestion (daily)	0 934	0 292	1 393	1 190	18 84
Excretion urine	0 099	0 096	0 742	1 006	17 60
„ faeces	0 683	0 136	0 437	0 135	1 10
„ total	0 782	0 232	1 179	1 141	18 70
Retention daily	0 151	0 060	0 214	0 049	0 14
„ per cent	16 2	20 6	15 3	4 1	0 7

### References

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## CHAPTER XXVI

### MISCELLANEA AND CONUNDRUMS

#### ARGYRIA

GENERALISED argyria is a rare condition nowadays. The patient's skin has a characteristic slate blue or leaden tint, due to the deposition of silver in the skin. Silver is also deposited in the liver, kidneys and elsewhere. Though the "pigmentation" of the skin is most marked in the exposed parts, it is unlikely that the action of light has any direct effect on the formation of the black deposit (metallic silver, or silver oxide). The condition was common in the middle of the last century. The silver was absorbed from the intestine as a rule, following prolonged oral administration of silver salts (for tabes, epilepsy, etc.), but occasionally the silver was absorbed from other sites (*e.g.*, from the vagina, following douches).

Localised argyria, or argyrosis (industrial, or due to local applications to the conjunctiva, etc.), is not uncommon at the present time.

As an extension of the clinical examination, histochemical investigation of a piece of skin obtained by biopsy is valuable. Unstained paraffin sections and sections lightly stained (*e.g.*, with methyl green and eosin) should be examined. The routine comparatively intense stains (*e.g.*, hæmatoxylin and eosin) may partially or completely mask the very fine granules of silver. The silver is deposited in the basement membranes of the sweat glands and sweat ducts, and to a lesser extent in the sebaceous glands and round the elastic fibres in the dermis. In one case examined by Dr Firth and the writer (*Brit J Derm Syph*, 1924, 36, 105), there was no deposit round the elastic fibres. The sweat glands and sweat ducts were readily picked out in unstained sections, owing to the deposit of silver in the form of extremely fine granules. The silver may be removed from the sections by taking them down the alcohols to water in the usual way, and then treating them with a mixture of equal parts of potassium ferri-cyanide (10 per cent) and sodium thio-sulphate (20 per cent) for fifteen minutes or longer, until the silver has been dissolved out. So far as the writer is aware, no other deposit in the skin situated in the sites mentioned would be removed by this chemical treatment. The silver may be even more readily removed by 2 per cent aqueous sodium or potassium cyanide solution, applied for about ten minutes.

#### BLOOD: DETECTION OF BLOOD STAINS ON CLOTHES, ETC

The examination of a stain for blood is generally undertaken by an expert in forensic medicine and toxicology, or by a public

analyst The chemical pathologist is occasionally requested to do this work, and the student is expected to know the general principles of the subject, wherefore a brief account is given here

If the suspected stain is on metal or any other hard surface, it is scraped off, and portions or the whole of the scrapings (depending on the amount) are examined for blood If it is on clothes, paper, etc., the stain is cut out, and the whole or portions of the material are treated as indicated below

The older methods consisted in the extraction of the stain or scrapings with distilled water, or physiological saline, or 1 per cent ammonia, or a cold saturated solution of borax containing a trace of ammonia, and so on The extract was then examined spectroscopically for hæmoglobin and its derivatives, or by means of the guaiac, benzidine, reduced phenolphthalein, etc., tests, or an attempt was made to prepare Teichmann's hæmin crystals Of recent years these methods have largely been discarded in favour of the preparation of hæmochromogen crystals in the presence of pyridine (see below), and the confirmation of the nature of the crystals spectroscopically, either directly with a microspectroscope or, after solution of the crystals, with an ordinary direct vision spectroscope The absorption bands of hæmochromogen are illustrated on p 212 (There is a very slight difference in the position of the bands of pyridine hæmochromogen and of globin hæmochromogen, but this cannot be detected by the ordinary hand spectroscope)

If a microspectroscope is available another simple method is to add one or two drops of concentrated sulphuric acid to the scrapings or stain placed on a microscope slide, cover and examine for the bands of acid porphyrin (for spectrum see p 212)

The ease of detection of blood simply depends on the amount, the methods used for blood in urine (Chapter X) or in gastric contents (Chapter XXII) serve also for stains which are relatively large, it is when the stain is small that there is difficulty, clearly it is necessary to make sure that negative results are not due merely to the effect of the dilution introduced in the test, and it follows that negative results should not be recorded until sensitive methods (cf p 212 for relative intensities of absorption bands) have been employed in which the dilution effect is small Naturally none of the chemical methods differentiate human blood from that of other animals

**Pyridine-Hæmochromogen Test** (cf Kerr and Mason, *Brit Med J*, 1926, 1, 134)

Treat the scrapings, or the stain which has been cut out of cloth etc., on a microscope slide with 2 or 3 drops of Takayama's solution 2 which consists of —

10 per cent sodium hydroxide	3 c c
Pyridine	3 "
Saturated aqueous glucose	3 "
Distilled water	7 "

Mount under a cover slip and examine with the microscope

If blood is present the colour changes through green brown and dark red to pink, and the characteristic salmon pink hæmochromogen crystals usually appear in one to six minutes. A negative result should not be recorded unless crystals are absent at the end of half an hour. The crystals (Fig. 83) are rhomboids or rod shaped, or tiny elongated rectangles with oblique ends, very similar in appearance to hæmin crystals but pink and not brown.

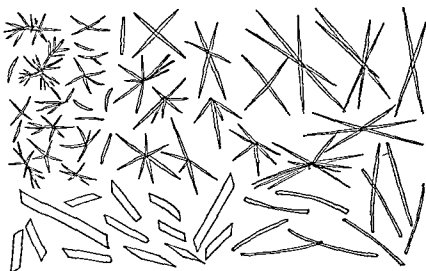


FIG. 83 Pyridine-hæmochromogen crystals

The NaOH dissolves the blood and liberates alkaline hæmatin, which is reduced by the glucose to alkaline reduced hæmatin and this combines with pyridine to give pyridine hæmochromogen.

If the test is performed at room temperature, as above described, the Takayama solution is less sensitive immediately after preparation than when it has been kept for two days or longer. If, however, the slide is heated till bubbles appear, crystals are formed almost at once even with fresh Takayama solution. Moreover, unlike the hæmin test there is no danger of over heating. The solution keeps for one or two months.

This method is more sensitive and simpler than Teichmann's hæmin test and gives positive results with blood stains that have been heated to 150° C, or that have been washed in hot water, or that are contaminated with rust.

#### GAUCHER'S DISEASE AND NIEMANN'S DISEASE

In both these diseases the spleen is enlarged, and paraffin sections of the spleen show large cells with spaces caused by solution of lipoids by the solvents used in the paraffin process. Frozen sections should therefore, be prepared as well as paraffin sections, unstained and stained sections should be examined under the polarising microscope. For a discussion of the different fat and

lipoid stains which may be used, the reader should consult histological manuals and the papers referred to below

From the chemical point of view, the spleen cells in Gaucher's disease are said to contain kersin, and there is no lipæmia and no hypercholesterolaemia. In Niemann's disease (or "lipoid histiocytosis") the deposits in the spleen consist of phosphatides, cholesterol, and cholesterol esters, and not of kersin, and there is typically lipæmia and hypercholesterolaemia (MacFate, R. P., *Arch Path*, 1928, 6, 1054)

For further information the following references should be consulted —

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### GOUTY TOPHI

In order to demonstrate that tophi are due to gout they are examined for crystals of sodium biurate. Occasionally, when the contents of the tophus are liquid, it is possible to aspirate enough fluid for microscopical examination with a needle and syringe. Usually, however, a small incision into the tophus is required, after which a smear may be made on a slide from the expressed juice. The smear is mounted under a cover slip with the addition of a drop of water if necessary, and examined with a  $\frac{1}{2}$  in objective for the characteristic acicular crystals of sodium biurate. If a polarising microscope is employed, it will be seen that the crystals are anisotropic (doubly refracting). So far as the writer is aware, anisotropic acicular crystals obtained from a tophus can only be sodium biurate.

The only swellings likely to be mistaken for gouty tophi are small sebaceous cysts or small tumours, neither of which yields acicular crystals.

### HÆMOCHROMATOSIS

The characteristic bronzing of the skin in this disease may or may not be associated with glycosuria. The glycosuria is due to cirrhosis of the pancreas, and if this is very extensive the glycosuria is accompanied by signs and symptoms of diabetes. Hence the older name "bronzed diabetes". The chemical examinations of the blood and urine in this type of diabetes are the same as those required in diabetes mellitus and have been considered previously in Chapters VII and VIII.

The diagnosis of hæmochromatosis may be established conclusively during life by examination of a piece of excised skin for iron. Paraffin sections are prepared in the usual way, care being

taken in the process to exclude gross dust, and reagents containing iron or acids. Alcohol is the best fixative. The sections are taken down the alcohols into water, and are then placed for twenty to thirty minutes in a freshly prepared 2 per cent solution of potassium ferrocyanide in 1 per cent v/v hydrochloric acid. They are then washed in distilled water, and counterstained with eosin, safranin, or neutral red. The preparation is completed in the usual way. Several modifications of the above technique are given in the histological text books. Thus the "iron reagent" may include ferricyanide (which yields ferrous ferricyanide or Turnbull's blue with ferrous iron) as well as ferrocyanide (which combines with ferric iron to form ferric ferrocyanide or Prussian blue). Organic iron (but not hæmoglobin) may be "unmasked" by preliminary treatment with acid alcohol (e.g., 3 per cent nitric acid in 95 per cent alcohol). In the writer's experience these modifications are unnecessary in the examination of skin sections in hæmochromatosis. Ammonium sulphide reacts with both ferric and ferrous iron, but is not specific for iron, since salts of silver, mercury and lead are also stained. The iron containing pigment in hæmochromatosis is called hæmosiderin, the chemical nature of which is not known with certainty, though it is commonly regarded as an oxide of ferric iron. Another pigment, hæmofuscin, which does not contain iron, according to some, or which contains only "masked" iron according to other workers, is often associated with hæmosiderin.

The iron reaction is most conspicuous in the dermis, and is often marked round the bases of the hair follicles and the sweat glands. The pigmentation of the skin in hæmochromatosis is caused partly by the deposition of hæmosiderin in the dermis, and partly by an excess of melanin in the deepest layers of the epidermis. According to Mallory *et al* (*J Med Research*, 1920-21, 42, 461), this excess of melanin may result from damage to the adrenals by the deposition of hæmosiderin therein.

It is generally considered that hæmochromatosis is due to an increased avidity of the tissues for iron. Sheldon (*Quart J Med*, 1927, 21, 123) found an excessive deposit of iron in all the tissues examined except the blood, which contained possibly slightly less than normal. Since the time taken for these deposits to accumulate is very long he suggests that possibly hæmochromatosis is an inborn error of metabolism, the accumulation of pigment being so slow that the characteristic clinical symptoms (pigmentation of skin, cirrhosis of liver, sclerosis of pancreas and resulting diabetes) do not appear until middle age.

As a result of metabolism experiments it has been found that there is some retention of food iron. Garrod *et al* (*Quart J Med*, 1913-14, 7, 129) failed to detect iron in the bile, faeces and urine. The quantity of iron in the blood of their case, and of other reported cases, is not increased according to modern standards.

Several other hypotheses as to the ætiology of hæmochromatosis have been advanced, for a discussion of which, together with fuller details of the disease, and an extensive bibliography, the reader is

referred to Sheldon's book (*Hæmochromatosis*. London H Milford, - Oxford University Press, 1935)

## SCLEREMA NEONATORUM

(Adiponecrosis subcutanea neonatorum)

Thus is an uncommon condition peculiar to infants, and is characterised by hard, well defined thickenings of the subcutaneous tissues. The diagnosis may be established during life by examination of a piece of excised skin and subcutaneous fat. Frozen sections show, in the subcutaneous fat, large numbers of sheaves of acicular crystals which are anisotropic (doubly-refracting), and which lie within fat globules and free. It has been concluded that these crystals consist of neutral fats, presumably stearin and palmitin. Histologically the picture suggests a primary chronic inflammation (possibly toxic in origin) with a patchy necrosis of fat, there is *giant cell formation and a varying degree of calcification*. Chemically (see table) there is a definite elevation of the melting point of the

### *Subcutaneous Fat in Health and in Sclerema*

(Channon and Harrison)

Sclerema case number	Age (weeks)	Melting points °C		Iodine values	
		Sclerema	Normal	Sclerema	Normal
I	6	50.5	—	42.1	—
IV	12	47.3	37.3	45.5	53.8
III	<div> <div>21 (biopsy)</div> <div>24 (autopsy)</div> </div>	<div> <div>38.5</div> <div>42.9</div> </div>	30.5 to 27.9	<div> <div>52.5</div> <div>54.0</div> </div>	56.9 to 60.6

extracted "fat," and an iodine value which is slightly, but not definitely significantly, lower than in healthy infants. There is also an increased amount of cholesterol, calcium and phosphorus. The chemical findings in sclerema, after due allowance has been made for the variations in the fat constants observed at different ages in infants, are compatible with the hypothesis that an inflammatory reaction is associated with a preferential absorption of olein, and hence a relative increase in the proportion of stearin and palmitin which crystallise out during life. An excess of cholesterol, calcium and phosphorus would be expected during the process of repair. There is no evidence of saponification of the fat, but there may be a very slight acid hydrolysis. The true skin is normal macroscopically and microscopically.

The above is a brief summary of the following articles —

- CHANNON, H. J., and HARRISON, G. A. *Biochem J.*, 1926, 20, 84.  
 GRAY, A. M. H. *Arch. Derm. and Syph.*, 1926, 14, 635.  
 HARRISON, G. A. and MCNEE, J. W. *Arch. Dis. Child.*, 1926, 1, 63 and 123.  
 KOHNSTAM, G. L. S., and HERBERT, F. K. *Arch. Dis. Child.*, 1927, 2, 349.



## XANTHOMATOSIS

Xanthoma nodules in the skin have a yellow colour which varies from bright yellow to reddish yellow, depending on the degree of hyperæmia. The yellow pigment is probably a lipochrome. The diagnosis is readily established by examination of an excised nodule. Histologically xanthoma tumours generally show characteristic 'foam cells' in paraffin sections. The spaces in these cells are caused by solution of lipoids in the paraffin process. In frozen sections either numerous doubly refracting (anisotropic) droplets or clusters of acicular crystals are seen when examination is made under the polarising microscope. With the Nicol's prisms crossed each droplet appears as a white Maltese cross on a black background. It is generally believed that these appearances are due to the presence of cholesterol esters. Certainly these tumours contain an excess of cholesterol (see table, p. 513).

There are two main types of cutaneous xanthomatosis, diabetic and non diabetic. In the diabetic variety suitable treatment with diet and insulin leads to the rapid disappearance of those nodules which have a good blood supply (Major, R. H., *Bull Johns Hopk Hosp* 1924, 35, 27). In the non diabetic variety insulin has no apparent effect either on the hypercholesterolaemia or on the xanthoma deposits (Ingram, J. T., *Brit J Derm Syph*, 1927, 39, 335). The writer was also unable to reduce the hypercholesterolaemia of a case of nephrosis with insulin (*Chem and Indust*, 1924 43, 1160). Whilst some patients with xanthomatosis are obviously diabetic, and others after most careful blood examination show no evidence of hyperglycaemia, yet others have no glycosuria on unrestricted diets but do have hyperglycaemia with or without transient glycosuria after glucose, and may, therefore, be regarded as potential diabetics (*Brit J Derm Syph*, 1923 35, 81, and Wile, U. J., Eckstein, H. C., and Curtis, A. C., *Arch Derm and Syph*, 1929, 20, 489). This third group is regarded by some workers as connecting the other two, but other investigators consider that true non diabetic xanthomatosis is quite a separate condition.

Hypercholesterolaemia is the rule in all types of xanthomatosis, but several cases have been reported in which a normal blood cholesterol was coincident with xanthoma nodules. It has been generally assumed that the finding of a normal blood cholesterol is due to persistence of the xanthoma lesions after the hypercholesterolaemia has disappeared. That the xanthoma deposits are due to an excess of circulating cholesterol, however, has not been conclusively proved, and recent writers have tended to give up this view.

For further information the reader is referred to Weber's paper (*Brit J Derm Syph*, 1924, 36, 335), and to the articles cited, most of which give extensive references.

*Analyses of a Xanthoma Nodule*<sup>2</sup>

	Xanthoma	Control <sup>1</sup>
Cholesterol as per cent of ether soluble matter	29.4	3.60
"    "    "    dried tumour	9.2	0.53
"    "    "    fresh tumour	2.8	0.16
"Total lipoids" (ether soluble matter) as per cent of dried tumour	31.3	14.8
"Total lipoids" (ether soluble matter) as per cent of fresh tumour	9.5	4.4

<sup>1</sup> The control estimations were performed on a piece of skin obtained post mortem from another patient of about the same age, who did not have xanthomatosis or diabetes. The skin included epidermis and dermis but only a trace of subcutaneous fat.

<sup>2</sup> *Proc Roy Soc Med*, 1924 18 (*Sect Dis Child*) 7

## CONUNDRUMS

Now and then the chemical pathologist is given interesting problems which may often be solved with the aid of the simple chemical and microscopical methods described in previous chapters, if interpreted in the light of the clinical findings. For instance, the recognition of pseudo calculi (Chapter IV), the confirmation of the presence of methylene blue in the urine by reduction with glucose (Chapter XI) and the Sudan III test for chyluria (Chapter XIV) provide examples.

The evidence from which it may be deduced that a sample of urine has been contaminated by faeces is given in Chapter IV. The reverse condition, viz., the contamination of faeces by urine, may be obvious to the naked eye, but if not obvious the following test may be applied.

Strain off the liquid from the faeces, or, select a semi-liquid portion of the stool and add about an equal volume of distilled water. Bring to the boil and filter. Test the filtrate for chlorides with nitric acid and silver nitrate (see Chapter II). Uncontaminated faeces contain only the merest trace of chlorides, wherefore only a slight opalescence will be observed. Faeces plus urine will give rise to a more or less dense precipitate of silver chloride.

Occasionally it is important to know whether the discharges from sinuses following operations in the region of the kidneys or bladder (e.g., for hernia, etc.) contain urine. The recognition of urine is generally simple. The colour or reaction, if acid, may lead to the suspicion of urine, but the diagnosis can generally be clinched by an estimation of urea. Thus in one case of this nature the urea amounted to 1.02 per cent. In another instance, the "exudation" from a herniotomy scar contained 2.4 per cent of urea. Since, however, the blood and other body fluids also contain urea, quantitative determinations are essential, and when the

sinus fluid does not contain a gross concentration of urea (as it did in the above cases), it is necessary to estimate simultaneously the urea in the blood. This aspect of the problem arose in a request to ascertain whether the fluid from a cyst, in a case of congenital cystic disease of the kidneys, contained urine or "cyst fluid." The results of examination were as follows—Colour, amber, reaction, slightly alkaline to litmus (the fluid had stood for several hours before examination), protein present in large quantities, urea, 0.41 per cent, chlorides as NaCl, 0.643 per cent, spectroscopically, no absorption bands. The colour pointed to the fluid containing urine, and both the urea and the chlorides were a little higher than would be expected in a cyst fluid, but without a knowledge of the blood urea, which might easily be 400 or 500 mgm per 100 c.c. (0.4 or 0.5 per cent) in such gross renal inefficiency, it would be unwise to state dogmatically that the fluid did contain urine.

One of the writer's diabetic patients skilfully substituted tap-water for the urine he was supposed to be supplying to his father for sugar tests, and explained the absence of colour by declaring that he was drinking a great deal! When analysed, the reputed urine contained no urea and a mere trace of chlorides.

Chemical examination of discharges from laparotomy wounds in the neighbourhood of the liver and pancreas may be valuable in deciding whether there is a leakage of pancreatic juice. An estimation of the concentration of diastase (Chapter XIII), and tests for trypsin<sup>1</sup> (see Chapter XXIV), usually provide a conclusive answer to the conundrum. The following is an example—"The fluid is blood stained, alkaline to litmus, contains trypsin, and 2,500 units of diastase per 1 c.c. Pancreatic juice is certainly present." The same problem may arise in cases of biliary fistula, viz., is pancreatic juice escaping as well as bile? The following result was obtained recently—"The fluid is deeply stained with bile, alkaline to litmus, contains trypsin, and 2,000 units of diastase per 1 c.c. There must be an escape of pancreatic juice as well as bile." Whilst discussing tests for enzymes, it is interesting to record that in the fluid from a fistula, which was suspected to originate from the parotid duct, 10,000 units of diastase were found per 1 c.c., thus confirming the suspicion.

At one time in a certain institution the new born babies all suffered from sore buttocks, which it was thought might be due to incomplete rinsing of the diapers after they had been washed in soda. This surmise was supported by the following simple observation. A diaper on its return from the laundry was soaked in a small quantity of water, which was then tested with red litmus paper, which turned deep blue!

It has more than once been suggested that in gross renal inefficiency there may be an actual excretion of urea and other bodies into the bowel, and that for this reason the vomiting in uræmia might in part be regarded as a compensatory mechanism.

<sup>1</sup> These discharges are alkaline, so no adjustment of reaction is necessary for the trypsin test. Physiological saline is used as control (cf p. 482).

In two cases, analyses of the blood and vomit did not support this hypothesis, the results being readily explicable as a simple diffusion of urea into the gastric, the intestinal and the other secretions poured into the bowel

	<i>Case I</i>	<i>Case II</i>
Blood urea, mgm per 100 c c	219	316
Urea in vomit, mgm per 100 c c	116	125
Ammonia in vomit, mgm per 100 c c	22	22
Urea in ascitic fluid, mgm per 100 c c	215	—

Occasionally it is necessary to decide whether fluid escaping from the nose, ear or a wound of the skull contains cerebrospinal fluid. The most useful tests are for chlorides (in normal cerebrospinal fluid 700 to 760, in normal serum 560 to 620 mgm per 100 c c), and for proteins (in normal cerebrospinal fluid 10 to 35, in normal serum 5,600 to 8,500 mgm per 100 c c), but if the discharge is grossly contaminated with blood, identification of cerebrospinal fluid is difficult or impossible.

The differentiation between serum and plasma may be a problem of importance. Thus the analyst may receive a fluid without information as to whether it be serum or plasma, or plasma may have been forwarded for a Wassermann reaction, and the problem is how to prepare serum from it, the identification of plasma likewise is useful as a class exercise to illustrate the difference between plasma and serum. The essential practical point is that if too much calcium chloride solution be added to plasma it will not clot, obviously there will be no clot if insufficient is added.

*The Recalcification of Plasma* To 1 c c of the fluid add 0.2 c c of a 5 per cent solution of crystalline  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$  (or 0.2 c c of 2.5 per cent anhydrous calcium chloride). Mix, set aside, and examine from time to time. If the fluid was oxalated or citrated plasma, in a few minutes the mixture will clot into a gel so that the tube can be inverted without loss of contents. Later the fibrin will contract and squeeze out the serum. Coagulation is accelerated if the test is performed at  $37^\circ \text{C}$ .

## APPENDIX

### REAGENTS. THEIR PREPARATION AND STANDARDISATION

**Acetic Acid** Glacial acetic acid contains 99 to 100 per cent of the acid S G 1.060

Acetic acid B P is 33 per cent w/w S G 1.044 to 1.045

An approximately N/1 solution may be prepared by diluting 60 c c of glacial acetic acid with distilled water to 1,000 c c (cf table on p 5)

**Acetone** B P, 56.5° C S G 0.796 to 0.801 Dry over calcium chloride

**Alcohol** See S G tables and B P of organic solvents When recovering alcohol distil from calcium oxide (quick lime)

**Alcoholic Soda** For differential estimation of "fat" in faeces, Chapter XXIV

An approximately 0.1 N solution may be prepared by dissolving 4.2 gm of stick caustic soda in about 10 c c of distilled water, and diluting to 1,000 c c with absolute alcohol The solution is allowed to stand for twenty four hours or longer, and is then accurately standardised against a 0.1 N solution of pure oxalic acid (*qv*), using phenolphthalein as indicator

**Ammonia Solution or Ammonium Hydroxide** Concentrated ammonia solution has a S G of 0.88 To make an approximately N/1 solution dilute 50 c c of concentrated ammonia solution with distilled water to 1,000 c c (cf table on p 5 and S G tables)

**Ammonium Molybdate Solution** See "Calculi," Chapter IV Dissolve 12.4 gm of crystalline ammonium molybdate ( $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ ) in distilled water without heating, and make up to 100 c c Alternatively a saturated solution in water may be used

Some samples of molybdate contain traces of phosphate, wherefore the following alternative solution is often employed Powder 7.5 gm of crystalline ammonium molybdate in a mortar, and then rub it up with 30 c c of concentrated ammonia (S G 0.88) When solution is complete, pour it gradually into a mixture of 90 c c of concentrated nitric acid (S G 1.42) and 40 c c of distilled water, cooling thoroughly during the addition Add 160 c c of distilled water, and filter if necessary On boiling 5 to 10 c c of the final solution a yellow precipitate should not be obtained If a yellow precipitate is noted, the main bulk of the final solution should be placed in the incubator at 37° C for several days, until there is no further precipitate of ammonium phosphomolybdate The clear and colourless supernatant fluid is then decanted

**Ammonium Sulphide (yellow)** For reduction of hæmoglobin

and derivatives (see Chapters X and XVIII) This is usually purchased

A colourless solution of ammonium sulphide can be made by saturating a 1 in 3 ammonia solution (1 part of concentrated ammonia, S G 0.88, and 2 parts of distilled water) with  $H_2S$ , and then further diluting the solution with a third of its volume of the 1 in 3 ammonia solution

**Ammonium Sulphocyanide or Thiocyanate.** For use in chloride determinations (see Chapter XXII) An approximately N/10 solution is prepared by dissolving about 8 gm of the salt in 1,000 c c of distilled water It must be standardised against N/10 silver nitrate Ammonium thiocyanate is hygroscopic and is decomposed by heat

**Aufrecht's Reagent.** For protein determinations (Chapters III and XX)

Picric acid	1.5 gm
Citric acid	3.0 "
Distilled water	to 100 c c

**Benedict's Qualitative Solution.** For "Sugar," see Chapter VI

Sodium citrate	173 gm
Sodium carbonate, anhydrous	100 "
Copper sulphate, crystalline	17.3 gm

Dissolve the citrate and carbonate in about 600 c c of distilled water in a large beaker, with the aid of heat Dissolve the copper sulphate in about 100 c c of distilled water in another vessel, and pour it with constant stirring into the carbonate citrate solution Transfer to a 1,000 c c volumetric flask Wash out both beakers with three or four portions of distilled water, and add the washings to the flask Mix well, cool to room temperature, and make up to 1,000 c c with distilled water Filter if not clear The solution keeps indefinitely

**Benedict's Quantitative Solution** See Chapter VI.

Sodium citrate	200 gm
Sodium carbonate, anhydrous	75 "
(or 200 gm of crystalline sodium carbonate, $Na_2CO_3 \cdot 10H_2O$ )	
Potassium thiocyanate	125 "

With the aid of heat, dissolve the above in about 600 c c of distilled water, filter and cool to room temperature

Dissolve exactly 18 gm of pure air-dried crystalline copper sulphate in about 100 c c of distilled water, and pour slowly into the above solution with constant stirring Transfer quantitatively to a 1,000 c c volumetric flask Add 5 c c of 5 per cent potassium ferrocyanide and make up to 1,000 c c with distilled water

Filter if necessary The solution keeps indefinitely

**Bial's Reagent for Pentoses** See Chapter VI

Orcinol	0.4 gm
Concentrated HCl, A R	200 c c
10 per cent ferric chloride	0.5 c c

This solution keeps for a few weeks only, and should be discarded when a precipitate forms

**Brom-cresol-green** See Table of Indicators

A 0.4 per cent solution is prepared by rubbing up 0.1 gm of the solid indicator in an agate mortar with 2.9 c c of N/20 NaOH. Transfer quantitatively to a 25 c c volumetric flask and add distilled water to the mark. For most purposes a 0.04 per cent solution is required, and is prepared by diluting the above 1 in 10 with distilled water.

**Brom-cresol-purple** See Table of Indicators and Chapter IX

Prepare a stock 0.4 per cent solution as described under "Brom cresol green," but use 3.7 c c of N/20 NaOH for each 0.1 gm of solid indicator. For routine purposes dilute to 0.04 per cent.

**Brom-phenol-blue** See Table of Indicators

A stock solution of 0.4 per cent is prepared, using 3 c c of N/20 NaOH for 0.1 gm. For general use as indicator dilute the stock solution 1 in 10.

**Brom-thymol-blue** See Table of Indicators

As before prepare a stock 0.4 per cent solution, using 3.2 c c of N/20 NaOH for 0.1 gm. Dilute 1 in 10 with distilled water to obtain a 0.04 per cent solution.

**Bromine Water**

Prepare a saturated solution by shaking a few drops of bromine with cold distilled water. Continue the addition of bromine till 2 or 3 drops remain in excess at the bottom of the bottle.

**Cleaning Solution** See "Dichromate Cleaning Fluid"**Congo Red** See Table of Indicators

Congo red	0.5 gm
Distilled water	90 c c
Alcohol (95 per cent or absolute)	10 "

Congo red paper may be prepared by pouring the above solution over a piece of filter paper, which is then hung up to dry, and afterwards cut into small strips.

**Cresol Red** See Table of Indicators

Prepare a stock 0.4 per cent solution as described under "Brom cresol green," but use 5.3 c c of N/20 NaOH for 0.1 gm of the solid indicator. For use as indicator dilute the above 1 in 20 with distilled water (0.02 per cent).

**Dichromate Cleaning Fluid**

Sodium or potassium dichromate	10 gm
Sulphuric acid, 25 per cent v/v	100 c c

**Ehrlich's Diazo Reagent** See Chapter XIV

**Solution A** A saturated, or 0.1 per cent, solution of sulphanilic acid in 5 per cent v/v hydrochloric acid

**Solution B** 0.5 per cent aqueous sodium nitrite

For use mix 50 parts of A and 1 part of B

**Esbach's Reagent** For Protein, see Chapter III

Picric acid	1 gm
Citric acid	2 "
Distilled water	to 100 c c

**Fehling's Solution** (B.P. Codev formula) For "Sugar," see Chapter VI

The same solution is used for both qualitative and quantitative tests

**Solution (i) —**

Crystalline copper sulphate	34.64 gm
Concentrated sulphuric acid	0.5 c c
Distilled water	to 500 c c

**Solution (ii) —**

Dissolve 176 gm of Rochelle salt (potassium sodium tartrate) with the aid of heat in 300 or 400 c c of distilled water. Add 77 gm of potassium hydroxide sticks and, when this has dissolved, cool and make up to 500 c c

For use take equal parts of (i) and (ii). The two solutions keep well, the mixed solution is stable for a few weeks only, 10 c c of the mixed solution are reduced by 0.05 gm of glucose

**Fouchet's Reagent** For bilirubin see Chapters II, XII and XXIV

Trichloroacetic acid	25 gm
Distilled water	100 c c
10 per cent ferric chloride	10 "

**Gunzburg's Reagent** For Free HCl see Chapter XXII

**Solution A** 10 per cent phloroglucin in absolute alcohol

**Solution B** 10 per cent vanillin in absolute alcohol

For use mix 2 parts of A and 1 part of B

**Hydrochloric Acid** See S.G. Tables and table on p. 5

Concentrated HCl (S.G. 1.16) contains 36.6 gm of the acid in 100 c c, and is about 10 N

To prepare an approximately N/10 solution dilute the concentrated acid 1 in 100 with distilled water. To determine its exact normality standardise against 0.1 N caustic soda

**Hypobromite Solution** See 'Sodium Hypobromite'



Table of Indicators (from Clark)

Name	pH Range.	Colour Change	
		Acid	Alkaline or less acid
Methyl violet . . . . .	0.1 to 3.2	Green	Blue
Meta cresol purple (acid range) .	0.5 to 2.5	Red	Yellow
Thymol blue (acid range) .	1.2 to 2.8	Red	Yellow
Tropæolin 00 . . . . .	1.4 to 2.6	Pink	Yellow
Topfer's reagent (di methyl- amino azo benzene) . . .	2.9 to 4.2	Red	Yellow
Brom phenol blue . . . . .	2.8 to 4.6	Yellow	Blue
Methyl orange . . . . .	3.1 to 4.4	Red	Yellow
Congo red . . . . .	3.0 to 4.5	Blue	Red
Brom-cresol-green . . . . .	3.8 to 5.4	Yellow	Green
Methyl red . . . . .	4.4 to 6.0	Red	Yellow
Propyl red . . . . .	4.8 to 6.4	Red	Yellow
Chlor-phenol red . . . . .	5.0 to 6.6	Yellow	Red
Brom cresol purple . . . . .	5.2 to 6.8	Yellow	Purple
Latmus (azolitmin) . . . . .	4.5 to 8.3	Red	Blue
Brom thymol blue . . . . .	6.0 to 7.6	Yellow	Blue
Neutral red . . . . .	6.8 to 8.0	Red	Yellow
Phenol red . . . . .	6.8 to 8.4	Yellow	Red
Rosolic acid . . . . .	6.9 to 8.0	Yellow	Red
Cresol red . . . . .	7.2 to 8.8	Yellow	Red
Meta cresol purple (alkaline range)	7.6 to 9.2	Yellow	Purple
Thymol blue (alkaline range) .	8.0 to 9.6	Yellow	Blue
Cresolphthalein . . . . .	8.2 to 9.8	Colourless	Red
Phenolphthalein . . . . .	8.3 to 10.0	Colourless	Red
Thymolphthalein . . . . .	9.3 to 10.5	Colourless	Blue
Alizarin yellow G. . . . .	10.1 to 12.1	Colourless	Yellow
Tropæolin 0 . . . . .	11.1 to 12.7	Yellow	Orange

**Iodine Preparation of an N/10 Solution** Dissolve 25 gm of pure potassium iodide in about 200 c c of distilled water Weigh accurately 12.692 gm of resublimed iodine in a stoppered weighing bottle, and transfer into about 150 c c of the iodide solution in a 1,000 c c volumetric flask Use the remainder of the iodide for washing the residual iodine out of the weighing bottle into the flask Shake well until the iodine has dissolved, and fill to the mark with distilled water Standardise against 0.1 N sodium thiosulphate (*qv*), using soluble starch as indicator, and store in a dark stoppered bottle

An approximately 0.1 N solution may be prepared by adding 12.7 gm of iodine to 200 c c. of 12.5 per cent potassium iodide, shaking till solution is complete, and diluting to 1,000 c c with distilled water This solution is further diluted about 1 in 5, for immediate use, as an indicator for starch in diastase estimations (see Chapter XIII)

*The tincture of iodine used for bilirubinuria* (see Chapter II) contains iodine 2.5, potassium iodide 2.5, distilled water 2.5, and alcohol to 100 parts

**Iron Alum** Used as indicator in chloride estimations (see Chapter XXII)

Either a 30 per cent or a saturated solution (see Chapter XXII) of ferric ammonium sulphate  $(\text{NH}_4)_2\text{SO}_4 \cdot \text{Fe}_2(\text{SO}_4)_3 \cdot 24\text{H}_2\text{O}$

**Kastle-Meyer Reagent.** See "Phenolphthalein"

**Lugol's Solution.** Chapter XXIV

Iodine	.	4 gm
Potassium iodide	.	6 "
Water	.	to 100 c c

**Mercuric Chloride** Chapter XVI

A saturated solution is about 8 per cent

**Methyl Orange.** See Table of Indicators

A 0.1 per cent aqueous solution

**Methyl Red.** See Table of Indicators

To obtain a 0.05 per cent solution, dissolve 0.1 gm of the solid indicator in 100 c c of absolute alcohol and dilute to 200 c c with distilled water

When estimating urea in blood (Chapter V) a saturated solution of methyl red in 50 per cent alcohol is used

**Nessler's Reagent** For Ammonia, see Chapter V

**Double Iodide Solution** Dissolve 150 gm of potassium iodide in 100 c c of distilled water Add 200 gm of mercuric iodide,  $\text{HgI}_2$ , and wait till solution is complete Then dilute to 1,000 c c with distilled water and filter Dilute the filtrate to 2,000 c c

**Ten per cent Sodium Hydroxide** Prepare a saturated solution of sodium hydroxide (about 55 per cent) by adding an excess of NaOH to about 200 c c of water, and stopper securely After two or three days decant the clear supernatant fluid and dilute with distilled water to 10 per cent (Add 45 c c of water to each 10 c c of supernatant fluid) Check the concentration of the sodium

hydroxide by further diluting 10 c c to 25 c c with distilled water, and titrating 10 c c of the supposed 4 per cent NaOH with N/1 acid. If the concentration differs from the theoretical by more than  $\pm 5$  per cent (i.e., if in the titration 10 c c of the sodium hydroxide require more than 10.5 c c or less than 9.5 c c of N/1 acid) it must be adjusted.

### *Preparation of Nessler Reagent*

10 per cent sodium hydroxide	700 c c
Double iodide solution	150 "
Distilled water	150 ,

**Nitric Acid** See table on p. 5

Concentrated nitric acid contains 99.1 gm of acid per 100 c c, and has a S.G. of 1.42

To prepare an approximately N/1 solution dilute 63 c c of the concentrated acid to 1,000 c c with distilled water

**Nylander's Reagent** For "Sugar" see Chapters II and VI

Dissolve 40 gm of Rochelle salt (sodium potassium tartrate) and 20 gm of bismuth subnitrate in 1,000 c c of 8 per cent sodium hydroxide

**Obermayer's Reagent** For Indican see Chapters II and XIV

Ferric chloride	1 gm
Concentrated HCl	500 c c

### **Oxalic Acid**

To prepare a N/10 solution dissolve 6.301 gm of the pure acid  $(\text{COOH})_2 \cdot 2\text{H}_2\text{O}$  in distilled water and make up to 1,000 c c

**Phenolphthalein** See Table of Indicators

For most purposes a 0.04 per cent solution in 95 per cent or absolute alcohol is suitable. When titrating the fatty acids in faecal extracts (Chapter XXIV) a more concentrated solution (e.g., 1 per cent in 50 per cent alcohol) is required.

**Phenolphthalin or Reduced Phenolphthalein** (Kastle Meyer reagent). For detection of blood in urine, faeces, etc. (see Chapters II, XXIV and XXVI)

Dissolve 2 gm of phenolphthalein and 20 gm of potassium hydroxide in 100 c c of distilled water. Add approximately 10 gm of zinc dust to the solution and boil until the pink colour has completely disappeared. Decant from the zinc and make up to 100 c c. Add a speck of zinc dust to keep the solution reduced and filter or centrifuge before use. The reagent becomes less sensitive with keeping.

**Phenol Red** See Table of Indicators

Grind up 0.1 gm of the solid indicator in an agate mortar with 5.7 c c of N/20 NaOH. Transfer quantitatively to a 25 c c volumetric flask and complete the volume by the addition of distilled water. This gives a stock 0.4 per cent solution. For

use dilute 1 in 10 (0.04 per cent), or 1 in 20 (0.02 per cent) with distilled water

**Phosphoric Acid.** The B.P. syrupy acid contains 89 (88 to 90) per cent w/w of  $H_3PO_4$ , SG about 1.75. Therefore 100 c.c. contain about 156 gm. of  $H_3PO_4$ , and as a tribasic acid the syrupy acid is about 48 N.

**Potassium Chromate.** As indicator in simple chloride determinations (see Chapter XX)

A 5 per cent aqueous solution

**Potassium Dichromate.**

A 0.1 N solution is prepared by weighing accurately 4.904 gm., dissolving it in distilled water and making up to 1,000 c.c.

**Potassium Ferricyanide** Recrystallisation of the salt for use in Hagedorn and Jensen's blood sugar method (see Chapter VII)

*Method I* Wash the crystals of the purest potassium ferricyanide that can be obtained commercially, with cold water. Dissolve the washed crystals in boiling water, and filter at once through an acid washed paper that has been previously treated with boiling water. Allow to crystallise in a covered vessel overnight in the ice room. Filter off the fine crystals on a Buchner funnel, and suck as dry as possible. Again recrystallise as above described. Finally, dry the crystals at 50° C. Keep the preparation away from sunlight throughout the process.

*Method II* (Fohn, O., *J. Biol. Chem.*, 1928, 77, 423) Add 100 gm. of the purest obtainable potassium ferricyanide to 400 c.c. of distilled water at 50° C. in a 1,000 c.c. beaker, and stir until solution is complete. Filter through a paper which has been previously well washed with distilled water, into a 2 litre vessel. Cool in running water.

To 600 c.c. of absolute alcohol add 0.1 c.c. of bromine and shake well. Add the brominated alcohol to the cooled ferricyanide solution, stirring well throughout the addition. The ferricyanide is precipitated at once. Filter without delay through a Buchner funnel in which rests a well fitting 9 cm. No. 50 Whatman paper. Wash the very fine precipitate in the funnel with 150 c.c. of alcohol to which have been added 2 or 3 drops of bromine. Then wash with 100 c.c. of ether similarly brominated, and finally with 25 c.c. of pure ether, applying strong suction to remove the ether as thoroughly as possible. Transfer the precipitate to a large clock-glass, and dry at about 50° C. Yield, 80 gm.

**Potassium Hydroxide** See table on p. 5 and S.G. Tables

To prepare an approximately 0.1 N solution, dissolve 5.8 gm. of pure stick potassium hydroxide in 1,000 c.c. of distilled water. Standardise against 0.1 N acid.

*Liquor potassæ B.P.* contains 5.0 gm. of KOH per 100 c.c.

**Potassium Permanganate** See also Chapter I

A 0.1 N solution is prepared by dissolving 3.162 gm. of pure dry  $KMnO_4$  in water, and diluting to 1,000 c.c. Standardise against 0.1 N oxalic acid (qv) or against pure dry sodium or potassium oxalate, in the presence of sulphuric acid at 60° C.

**Potassium Thiocyanate** (sulphocyanide) For use in chloride estimations (Chapter XXII)

An approximately N/10 solution is prepared by dissolving about 10 gm of the salt in 1,000 c c of distilled water. It must be standardised against 0.1 N silver nitrate. It is hygroscopic and is decomposed by heat.

**Seliwanoff's Reagent.** For Fructose (lævulose), see Chapter VI

Resorcin	.	.	0.1 gm
Concentrated HCl	.	.	66 c c
Distilled water	.	.	to 200 „

The solution keeps for a few weeks only, and must be discarded when a precipitate forms.

**Silver Nitrate.** For Chloride Determinations, see Chapters XIX, XX and XXII

A 0.1 N solution is prepared by dissolving 16.989 gm of the A.R. salt in distilled water and making up to 1,000 c c.

**Sodium Chloride** See also Chapter I

Physiological, or isotonic, or "normal" saline contains 0.85 gm of NaCl in 100 c c.

A N/1 solution of NaCl contains 5.845 gm per 100 c c.

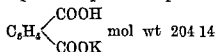
**Sodium Hydroxide** See also table on p. 5 and S.G. Tables

An approximately N/10 solution is prepared by dissolving 4.2 gm of stick NaOH in 1,000 c c of distilled water. This may be standardised against a 0.1 N acid.

For accurate work standard sodium hydroxide should be free from carbonate. The following method is recommended (see Cole's *Practical Physiological Chemistry*), and forms a convenient basis for the subsequent preparation of most of the standard acids and bases used in the laboratory. For an alternative preparation from  $\text{Ba}(\text{OH})_2$  and  $\text{Na}_2\text{SO}_4$  see Kay, W. W., and Sheehan, H. L., *Biochem. J.*, 1934, 28, 1795.

Dissolve 100 gm of the best NaOH in 100 c c of distilled water in a conical flask of resistance glass. Cover the mouth of the flask with tinfoil, and allow to stand overnight or longer for the carbonate to settle. Cut a No. 50 Whatman paper so that it is slightly too big to fit a Buchner funnel. Place the paper in a large beaker and wash it in turn with warm concentrated (1.1) NaOH, absolute alcohol, 50 per cent alcohol, and, finally, with large volumes of distilled water. Place the washed paper in the Buchner funnel, and apply gentle suction to fix it in place and to remove the greater part of the water. Now pour the concentrated alkali upon the middle of the paper, spread it with a glass rod and filter under suction. Dilute the clear filtrate quickly with cold distilled water, which has been recently boiled to remove  $\text{CO}_2$ , to make approximately N/1 NaOH (usually about 50 c c has to be diluted to 1 litre). Withdraw 10 c c and standardise roughly against N/1 HCl. Then dilute further with  $\text{CO}_2$  free water till it is approximately of the desired normality (e.g., 0.1 N). Transfer without delay to a suitable vessel, to which access of  $\text{CO}_2$  is prevented (e.g., apparatus illustrated

on p 78) Next day standardise the solution accurately against weighed amounts of pure acid potassium phthalate,

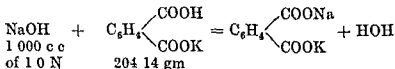


If the sodium hydroxide is about 0.1 N, weigh out accurately the phthalate (about 0.3 gm is suitable) and suspend it in about 20 c c of distilled water in a boiling tube. Titrate with the alkali in a current of  $\text{CO}_2$  free air (see Fig on p 78), using phenolphthalein as indicator. As the titration proceeds the phthalate completely dissolves.

If  $p$  be the weight of phthalate,  $s$  the volume of caustic soda required, and  $n$  the normality of the alkali,

$$n = \frac{1,000 \times p}{204.14 \times s}$$

This formula is arrived at as follows —



$$1 \text{ c c of } 1.0 \text{ N NaOH} = \frac{204.14}{1,000} \text{ gm of phthalate,}$$

$$s \text{ c c of } 1.0 \text{ N } \quad \quad = \frac{204.14 \times s}{1,000} \text{ gm } \quad \quad ,$$

$$s \text{ c c of } n \text{ N } \quad \quad = \frac{204.14 \times s \times n}{1,000} \text{ gm } \quad \quad ,$$

But  $s$  c c of  $n$  N caustic soda corresponded to  $p$  gm of phthalate

$$p = \frac{204.14 \times s \times n}{1,000}$$

or 
$$n = \frac{p \times 1,000}{204.14 \times s}$$

*Example* — Weight of phthalate was 0.3 gm

Volume of alkali required was 14.5 c c

Therefore normality of alkali was

$$\frac{1,000 \times 0.3}{204.14 \times 14.5} = 0.101$$

The caustic soda was 0.101 N

**Sodium Hypobromite** (For Urea, see Chapter V)

1 part of bromine . . . . . (2.5 c c ampoule)  
10 parts of 40 per cent NaOH . . . . . (25 c c)

The solution must be prepared on the day of the test

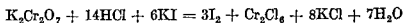
**Sodium Thiosulphate** For Iodometric Titrations (see "Blood-sugar Methods," Chapter VII)

To prepare an approximately N/10 solution dissolve 26 gm of

crystalline sodium thiosulphate,  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ , in 1,000 c c of freshly boiled distilled water. Standardise two or three days later by one of the methods given below. "Thio" solutions should be stored in dark stoppered bottles.

**Method I** Standardisation against 0.1 N potassium dichromate (q.v.)

Place 20 c c of N/10 potassium dichromate in a 500 c c conical flask. Add 10 c c of 10 per cent potassium iodide, 5 c c of concentrated HCl, and about 200 c c of water. The acid dichromate oxidises some of the iodide to iodine, and the amount of iodine formed is exactly proportional to the quantity of potassium dichromate used.



Titrate with the approximately 0.1 N sodium thiosulphate from a burette until the yellow colour of the liberated iodine has almost disappeared (about 15 c c). Add a few drops of 1 per cent soluble starch and continue the titration. The colour changes from a dirty dark yellow (mainly iodine) through a dirty dark green blue (blue starch iodide plus green  $\text{Cr}_2\text{Cl}_6$ ) to a clear bright pale green or green (chromium chloride alone). The end point is very sharp, and is easily recognised once it has been seen.

Perform a blank test, to allow for traces of iodate in the potassium iodide, as follows.—To 10 c c of the 10 per cent iodide, add 5 c c of concentrated HCl and a few drops of the soluble starch. Titrate with the thiosulphate. Subtract the blank value from the titration figure obtained for the 20 c c of dichromate.

**Example of Calculation** Twenty c c of 0.1 N dichromate required 19.2 c c of the thiosulphate. The blank was 0.2 c c of the thiosulphate. Therefore  $19.2 - 0.2 = 19$  c c of the thiosulphate are equivalent to 20 c c of 0.1 N dichromate. Since 20 c c of 0.1 N thiosulphate correspond to 20 c c of 0.1 N dichromate, to each 19 c c of the above thiosulphate 1 c c of  $\text{CO}_2$  free distilled water must be added to make it exactly 0.1 N.

Having suitably diluted the thiosulphate, it should be checked against the 0.1 N dichromate as above described, to make certain that it is accurately 0.1 N.

**Method II** Standardisation against acid potassium iodate (or against "neutral" potassium iodate).

Prepare a decinormal solution of acid or of "neutral" potassium iodate. Weigh exactly 0.3249 gm of  $\text{KH}(\text{IO}_3)_2$  (or 0.3568 gm of  $\text{KIO}_3$ ), and dissolve it in about 50 c c of distilled water in a beaker, if necessary heating gently. Transfer quantitatively to a 100 c c volumetric flask and dilute to the mark with distilled water.

Place 25 c c of the solution in a conical flask, and add 1 gm of potassium iodide dissolved in a little water, and a few c c of 10 per cent v/v hydrochloric acid. Titrate without delay with the approximately N/10 thiosulphate, using starch as indicator. Having thus determined the titre of the thiosulphate, dilute suitably to make exactly 0.1 N.

**Method III** Standardisation of N/200 thiosulphate using potassium iodate and the reagents employed in Hagedorn and Jensen's blood sugar method (see Chapter VII)

Dilute 5 c.c. of the approximately N/10 thiosulphate (26 gm per litre, see above) to 100 c.c. in a volumetric flask, or dissolve 0.65 gm of crystalline sodium thiosulphate in 500 c.c. of water

Prepare an N/200 solution of potassium iodate by weighing accurately 0.0892 gm of pure dry  $\text{KIO}_3$  and dissolving it in 500 c.c. of distilled water. This solution keeps for a considerable time

To 2 c.c. of the iodate add 3 c.c. of Hagedorn and Jensen's iodide chloride sulphate solution, and 2 c.c. of 3 per cent acetic acid. Titrate the liberated iodine with the thiosulphate, using 2 drops of the 1 per cent soluble starch in saturated sodium chloride as indicator

Two c.c. of 0.05 N potassium iodate correspond to 2 c.c. of 0.05 N thiosulphate. If the titration is not exactly 2 c.c., either introduce the appropriate factor into the calculation, or dilute the thiosulphate till it is exactly 0.05 N. Sodium thiosulphate of this concentration is stable for one or two days only

**Sodium Tungstate** For precipitation of blood proteins in the presence of acid (Chapter XIX) or of milk proteins (Chapter XXI)

A 10 per cent solution of  $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$  is used. Not all brands of sodium tungstate are satisfactory. The A.R. tungstate should be easily soluble in cold water and the solution should be alkaline to phenolphthalein. If not the product consists of complex tungstates (paratungstates). Folin states that such preparations can be rendered serviceable as follows—Dissolve 10 gm of the tungstate in 100 c.c. of water with the aid of heat and cool. Titrate 25 c.c. of the solution with 10 per cent sodium hydroxide, using phenolphthalein as indicator, until a pink colour persists for three minutes. Calculate the amount of 10 per cent sodium hydroxide required for larger quantities (e.g., 100 gm) of tungstate, and introduce that amount when preparing the tungstate solutions

It is convenient to prepare a stock of 2,000 c.c. or more of 10 (or 20) per cent sodium tungstate, which is alkaline to phenolphthalein. On standing a deposit forms which contains calcium. The clear supernatant fluid is decanted for use

**Soluble Starch** Used as indicator in iodometric titrations (see "Blood sugar Methods" in Chapter VII), and as substrate in diastase estimations (Chapter XIII)

**AS INDICATOR** (a) *One per cent in "saturated" sodium chloride* Make 1 gm of soluble starch into a paste with about 10 c.c. of saturated NaCl and pour into about 80 c.c. of the saturated chloride brought to the boiling point. Cool the mixture, transfer to a volumetric flask or measuring cylinder with a few cubic centimetres of water and make up to 100 c.c.

(b) *One per cent in Water* Shake up 1 gm of soluble starch with about 1 c.c. of water in a test tube, and pour into about 70 c.c. of boiling water. Wash out the tube with a few more cubic



centimetres of water and add to the boiling solution. Cool, and make up to 100 c c.

Divide the solution into 10 to 12 portions, placing each in a test tube plugged with cotton wool. Sterilise by heating in a boiling water bath for half an hour on each of three successive days. Finally cover each plug with a rubber cap to prevent evaporation.

**FOR DIASTASE ESTIMATIONS** A stock solution of 2 per cent soluble starch in 10 per cent sodium chloride is diluted 1 in 20 with distilled water, thus giving a test solution of 0.1 per cent soluble starch in 0.5 per cent NaCl (see Chapter XIII for further details).

**Stokes' Reagent.** For reduction of hæmoglobin and its derivatives (see Chapters X and XVIII).

Dissolve 3 gm of ferrous sulphate in cold water. Dissolve 2 gm of tartaric acid in a little water, and add the solution to that of the ferrous sulphate. Add distilled water to 100 c c.

For use take about 5 c c of the above and add concentrated ammonia drop by drop, until the precipitate first produced is dissolved. Ammonium ferrotartrate is formed, which is a good reducing agent, but which rapidly absorbs oxygen from the air, and therefore must be freshly prepared.

**Sulphuric Acid** See S G Tables and table on p. 5.

One hundred c c of concentrated sulphuric acid (S G 1.84) contain 175.9 gm of the acid.

An approximately normal solution may be prepared by diluting 28 c c of the concentrated acid to 1,000 c c with distilled water. To determine its exact normality standardise against 1.0 N. NaOH.

**Thymol Blue** See Table of Indicators.

This indicator has both an acid and an alkaline range. This is taken advantage of in the acid determinations in gastric analysis (see Chapter XXII). For gastric work a 0.1 per cent solution of the indicator is convenient.

For general use as an indicator a 0.04 per cent solution is generally employed. To prepare a stock 0.4 per cent solution grind 0.1 gm of the solid indicator in an agate mortar with 4.3 c c of N/20 NaOH. Transfer quantitatively to a 25 c c volumetric flask, and add distilled water to the mark. The other two solutions mentioned above (0.1 and 0.04 per cent) are prepared by dilution of the stock solution with distilled water.

**Töpfer's Reagent.** See Table of Indicators and Chapter XXII.

Dimethylaminoazobenzene.	.	.	0.5 gm
Alcohol, 96 per cent	.	.	to 100 c c

**Uffelmann's Reagent.** For lactic acid (Chapter XXII).

To 50 c c of 2 per cent carbolic acid, add 1 or 2 drops only of 10 per cent ferric chloride, i.e., add just sufficient  $\text{FeCl}_3$  solution to produce a purple colour. The solution must be freshly prepared, for it is stable for only a few minutes.

## METHOD FOR CLEANING MERCURY

The soiled mercury is placed in a stout, wide mouthed bottle or filter flask, and a tube connected to a water tap is arranged so that its lower end dips under the surface of the mercury. By passing a strong stream of water through the mercury, coarse dirt, etc., will be washed away (Fig 84). The water is poured off, and the mercury

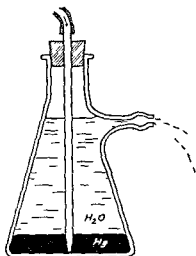


FIG 84 Washing mercury with a strong stream of tap water

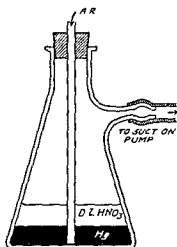


FIG 85 Apparatus for agitating dirty mercury with dilute  $\text{HNO}_3$  by drawing air through it  
The flask is the same as in Fig 84 but is connected differently

is then passed through the purifier (Fig 86). Should the mercury be grossly contaminated it is advisable first to agitate it with 5 per cent nitric acid contained in a filter flask, by drawing air rapidly through the mercury and acid for an hour or so (Fig 85). Then proceed with the washing as above.

The purifier (Fig 86) consists of a stout glass tube 100 cm long and about 2 cm in diameter, held vertically by clamps on a retort stand. The lower end of the tube is closed by a rubber bung through which is passed a piece of manometer tubing bent to form a syphon, the vertical limb being 13 cm long and supported by a cork on the base of the stand, or the apparatus may be blown in one piece (see Fig 86). Enough clean mercury is poured into the tube to form a layer 5 cm high, and 5 per cent nitric acid is added until the tube is filled to a height of 90 cm. A glass funnel, about 6 cm in diameter and connected by means of a short piece of rubber pressure tubing to a very fine glass jet, is placed in the open end of the tube, the jet just dipping below the surface of the nitric acid. The dirty mercury is poured into the funnel and falls from the glass jet as a stream of minute droplets, through the nitric acid, and accumulates at the bottom of the tube. The mercury in the syphon consequently rises, and overflows into a clean vessel placed to catch it. Since

the syphon leads out from the very bottom of the upright tube, there is always a column of 4 or 5 cm of mercury in the tube

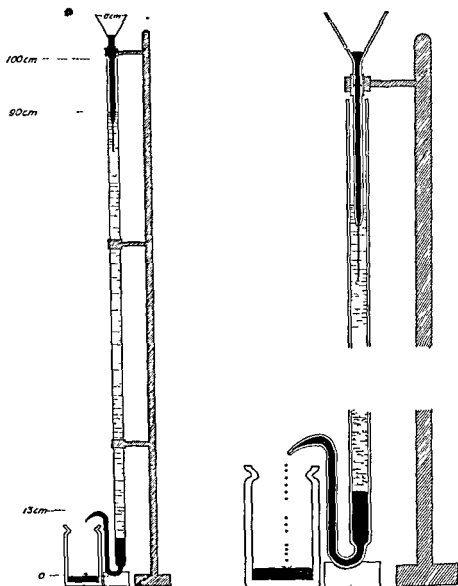


FIG 86 Mercury purifier For description see text

Mercury combines with the nitric acid to form a saturated solution of mercurous nitrate, which in turn reacts with metallic impurities in the falling droplets, and dissolves them out as nitrates

Finally the mercury is placed in a filter flask and washed, until free from acid, with tap water as described at the beginning of the technique (Fig 84), it is then shaken with distilled water and separated. To get rid of the last traces of moisture, when this is necessary, it is squeezed through chamois leather

## METHODS FOR CLEANING GLASSWARE

If the vessel contains infective material, such as faeces, it is advisable to add lysol or some strong disinfectant, and then to wash the contents down the lavatory pan

*Small glass articles*, such as test tubes, small beakers, and funnels, etc., are placed in a large enamel wash bowl, and boiled for a short time with approximately 10 per cent washing soda. They are allowed to cool and rinsed with tap water. They are then placed in another bowl (preferably porcelain) containing approximately 5 per cent commercial hydrochloric acid. This will neutralise any soda remaining on the articles. They are then thoroughly rinsed with tap water, and finally with distilled water, and either allowed to drain, or else dried in a hot air oven at a temperature just above  $100^{\circ}\text{C}$ , wire baskets should be lined with pieces of clean paper on which the inverted tubes, etc., are rested

The soda and acid will suffice for quite a large quantity of glassware, but the acid must be discarded as soon as it gives no effervescence with a few drops of the soda solution

In isolated instances boiling with washing soda will not remove all "dirt". In such cases the effect of concentrated hydrochloric acid should be tried. If this is unsuccessful the vessel should be filled with dichromate cleaning fluid (see "Reagents"), allowed to stand for a few days, and then treated as above. (If much grease is present, this should be removed with old methylated spirit, or "spirit residues," before boiling with soda.)

*Larger apparatus*, such as large beakers and flasks, may be cleansed by thorough brushing with some form of soap powder, and then rinsing with tap water, dilute acid, tap water, and finally distilled water

In all cases a liberal supply of tap water and distilled water should be used

For a method of cleaning and drying pipettes, see Chapter I

## CALIBRATION OF PIPETTES, Etc

This has been discussed in Chapter I fairly thoroughly, but the following practical information may be of service

Blood pipettes (0.1 to 1.0 c.c.) made "to contain" must be calibrated with mercury. Once, however, the accuracy of one pipette of a given capacity has been established by weighing mercury, other pipettes of the same capacity may be checked against it by introducing the same volume of mercury. The procedure is as follows—Select a piece of glass tubing about 3 in. long and with a bore of about  $\frac{1}{8}$  in. Seal one end in the flame in such a way that the bottom of the resulting tube is fairly flat, and has an area a little larger than the tip of the pipette which is to be calibrated. In another clean tube place 1 or 2 c.c. of clean mercury. Fit a test to the end of the first or standard pipette, and suck up mercury exactly

to the mark. Remove the pipette without loss or gain of mercury, and deliver the mercury into the specially prepared tube, which must have been thoroughly cleaned previously. Place a piece of white paper on the bench and a grease pencil between the teeth. Hold the special tube with its contained mercury in one hand obliquely above the white paper. Grip the top of the tube with the fingers so as not to heat the mercury (Neglect of this precaution, however, introduces no significant error, see later). With the other hand take up a second pipette, to which has previously been attached a teat. Suck up all the mercury into this second pipette without including any air bubbles, and in such a way that the lower end of the column of mercury lies exactly at the tip of the pipette. Mark the level of the top of the mercury column with the grease pencil held in the teeth. Return the mercury to the tube. Take up the mercury again in the standard pipette to insure that none has been lost. Make a permanent mark on the second pipette with a glass knife, or with fluoride marking fluid, to replace the grease pencil mark.

The method is much simpler to perform than to describe. With a little practice in the manipulation of the teat, and given *clean* tubes, pipettes, and mercury, the technique is easily acquired. It is not difficult, after a little experience, to calibrate ten or twelve pipettes in this way in an hour.

To check the capacity of pipettes by weighing water, proceed as follows.—Select two small conical flasks of the same size, and as light as possible. Place one on each of the pans of a balance, and add the necessary weights, or pieces of paper, etc., to balance them accurately. Fill the pipette to the mark with distilled water and deliver (either by touching off against the inside of the flask, or by blowing out, according to the type of pipette, cf Chapter I) the water into the flask. Determine the weight of water without delay.

Weight in gm of 1 c c of distilled water at different temperatures (Kaye and Laby) —

Temp °C	10	12	14	16	18	20
Weight in gm	0.99973	0.99953	0.99927	0.99897	0.99862	0.99823

Weight in gm of 1 c c of mercury at different temperatures (Kaye and Laby) —

Temp °C	10	12	14	16	18	20
Weight in gm	13.5708	13.5659	13.5609	13.5560	13.5511	13.5462

For ordinary purposes a pipette of which the capacity is the theoretical  $\pm 1$  per cent may be accepted. It is obvious, therefore,

that when calibrating by weighing water, the slight alterations in the volume of 1 gm of water with changes in temperature may be neglected (as also the expansion of the glass with rise in temperature) In other words, no significant error is introduced by presuming that 1 c c of water weighs 1 gm at the usual temperatures of the laboratory

Similarly the error which will ordinarily be introduced by neglecting the expansion of mercury with rise in temperature, though greater than in the case of water, will still not be significant, unless a greater accuracy than  $\pm 1$  per cent is desired At ordinary laboratory temperatures, therefore, the factor 1 c c of mercury weighs 13.56 gm may be utilised

### WHATMAN'S FILTER PAPERS

The purposes for which the different grades of filter paper are best employed are conveniently summarised by the manufacturers in the table which follows —

	Qualitative use where Ash Weight unimportant.	Washed in HCl Use for rougher routine gravimetric work	Washed in HCl and HF Use for gravimetric analysis	Thin Washed in HCl and HF For finest work	Toughened
Gelatinous or large particle precipitates	4	31	41	—	54
General work	1 or 2	30	40	44	52
Fine precipitates	3 or 5	32	42	44	50
Caustic solutions	3 or 5	—	—	—	} 54 52 50
Buchner funnels	5 or 1	32	42	—	

NOTE. No 43 Whatman paper which is fat free, is used in fat analyses

### CONVERSION FACTORS AND READY RECKONERS

Several ready reckoners have been given in the text (*e g*, for the conversion of the thiosulphate deficiency into mgm of glucose per 100 c c of blood in iodometric methods of estimating blood-sugar (Chapter VII), and for the conversion of the CO<sub>2</sub> evacuated into the "alkali reserve" expressed as volumes of CO<sub>2</sub> per 100 c c of plasma (Chapter IX) Other useful ready reckoners and conversion factors are collected together overleaf

## Gastric Analysis

Conversion of c.c. N/10 per cent. (Acid or Chloride) into gm. per 100 c.c. (as HCl). (1 c.c. of N/10 HCl per cent. equals 0.00365 gm. of HCl per 100 c.c.)

C.c. N/10 per cent	Gm per cent.	C.c. N/10 per cent	Gm per cent	C.c. N/10 per cent	Gm per cent	C.c. N/10 per cent	Gm per cent
1	0.004	31	0.113	61	0.223	91	0.332
2	0.007	32	0.117	62	0.226	92	0.336
3	0.011	33	0.120	63	0.230	93	0.339
4	0.015	34	0.124	64	0.234	94	0.343
5	0.018	35	0.128	65	0.237	95	0.346
6	0.022	36	0.131	66	0.241	96	0.350
7	0.026	37	0.135	67	0.245	97	0.354
8	0.029	38	0.139	68	0.248	98	0.358
9	0.033	39	0.142	69	0.252	99	0.361
10	0.037	40	0.146	70	0.256	100	0.365
11	0.040	41	0.149	71	0.260	101	0.369
12	0.044	42	0.153	72	0.263	102	0.373
13	0.047	43	0.157	73	0.266	103	0.376
14	0.051	44	0.161	74	0.270	104	0.380
15	0.055	45	0.164	75	0.274	105	0.383
16	0.058	46	0.168	76	0.277	106	0.387
17	0.062	47	0.172	77	0.281	107	0.391
18	0.066	48	0.175	78	0.285	108	0.394
19	0.069	49	0.179	79	0.288	109	0.398
20	0.073	50	0.183	80	0.292	110	0.402
21	0.077	51	0.186	81	0.296	111	0.405
22	0.080	52	0.190	82	0.299	112	0.409
23	0.084	53	0.193	83	0.303	113	0.412
24	0.088	54	0.197	84	0.307	114	0.416
25	0.091	55	0.201	85	0.310	115	0.420
26	0.095	56	0.204	86	0.314	116	0.423
27	0.099	57	0.208	87	0.318	117	0.427
28	0.102	58	0.212	88	0.321	118	0.431
29	0.106	59	0.216	89	0.325	119	0.435
30	0.110	60	0.219	90	0.329	120	0.438

**Conversion Factors for Substances commonly expressed  
in more than one way**

To convert—	Multiply by—	To convert—	Multiply by—
Acetone into Aceto-acetic acid	102/58 or 1 76	Aceto-acetic acid into acetone	58/102 or 0 57
$\beta$ -hydroxybutyric acid	104/58 or 1 79	$\beta$ hydroxybutyric acid into acetone	58/104 or 0 56
Ca into CaO	56/40 or 1 40	CaO into Ca	40/56 or 0 71
Mg MgO	$\frac{40 \cdot 3}{24 \cdot 3}$ or 1 66	MgO Mg	$\frac{24 \cdot 3}{40 \cdot 3}$ or 0 603
Na $\text{Na}_2\text{O}$	62/46 or 1 35	$\text{Na}_2\text{O}$ Na	46/62 or 0 74
K $\text{K}_2\text{O}$	94/78 or 1 21	$\text{K}_2\text{O}$ K	78/94 or 0 83
Cl NaCl	$\frac{58 \cdot 5}{35 \cdot 5}$ or 1 65	NaCl Cl	$\frac{35 \cdot 5}{58 \cdot 5}$ or 0 607
P $\text{P}_2\text{O}_5$	142/62 or 2 29	$\text{P}_2\text{O}_5$ P	62/142 or 0 44
P $\text{H}_3\text{PO}_4$	98/31 or 3 16	$\text{H}_3\text{PO}_4$ P	31/98 or 0 316
S $\text{SO}_2$	80/32 or 2 50	$\text{SO}_2$ S	32/80 or 0 40
S $\text{H}_2\text{SO}_4$	98/32 or 3 06	$\text{H}_2\text{SO}_4$ S	32/98 or 0 33
N Protein	6 25	Protein N	0 16
Ammonia N Ammonia	17/14 or 1 21	Ammonia Ammonia N	14/17 or 0 82
Creatine N Creatine	131/42 or 3 12	Creatine Creatine N	42/131 or 0 32
Creatinine N Creatinine	113/42 or 2 7	Creatinine Creatinine N	42/113 or 0 37
Urea N Urea	60/28 or 2 14	Urea Urea N	28/60 or 0 47
Uric acid N Uric acid	168/56 or 3 0	Uric acid Uric acid N	56/168 or 0 33

**Conversion of mgm per 100 c c into millimols, etc**

*Symbols Used* M, mols per litre mM, millimols per litre  
m Eq, milliequivalents per litre

**I Conversion of mgm per 100 c c into millimols per litre, and vice versa**

$$(a) \text{ mM} = \frac{10 \times \text{mgm per 100 c c}}{\text{molecular weight}} \left( = \frac{10\,000 \times \text{gm per 100 c c}}{\text{mol wt}} \right)$$

$$(b) \text{ mgm per 100 c c} = \frac{\text{mM} \times \text{mol wt}}{10}$$

*Examples* "357 mgm of Cl per 100 c c," is the same as "100 6 millimols of Cl per litre"

"588 5 mgm of NaCl per 100 c c," is the same as "100 6 millimols of NaCl per litre"

**II. Conversion of c c of Gas (at S T.P.) per 100 c c. into millimols per litre, and vice versa**

$$(a) \text{ mM} = \frac{\text{c c per 100 c c}}{2 \cdot 24}$$

$$(b) \text{ c c per 100 c c} = 2 \cdot 24 \times \text{mM}$$

*Examples* "70 8 c c of  $\text{CO}_2$  per 100 c c," is the same as "31 6 millimols of  $\text{CO}_2$  per litre"

"17 c c of  $\text{O}_2$  per 100 c c," is the same as "7 6 millimols of  $\text{O}_2$  per litre"



III. Conversion of mgm per 100 c c. into milliequivalents per litre, and *vice versa*

$$(a) \text{ m Eq} = \frac{10 \times \text{mgm per 100 c c} \times \text{valency}}{\text{mol wt}}$$

$$(b) \text{ mgm per 100 c c} = \frac{\text{m Eq} \times \text{mol wt}}{10 \times \text{valency}}$$

*Example* (taken from Gamble, Ross and Tisdall, *J Biol Chem*, 1923, 57, 637, and Gram's paper, *Am J Med Sc*, 1924, 168, 511)

Average acid base composition of venous plasma

Kations	mgm. per 100 c c	m Eq per litre	Anions	m Eq per litre
Na	330	143.4	HCO <sub>3</sub> (60 c c of bound CO <sub>2</sub> per 100 c c)	27.0
K	20	5.1	Cl (365 mgm per 100 c c)	103.0
Ca	10	5.0	HPO <sub>4</sub> (5 mgm of inorganic P per 100 c c)	3.0
Mg	3	2.5	SO <sub>4</sub> (1 mgm of inorganic S per 100 c c)	1.0
			Organic acids (assumed)	2.0
			Proteins (by difference)	20.0
Sum		156.0		156.0

"The ammonium value is too small to be considered . . .  
Physiological valency Ca 2, Mg 2, SO<sub>4</sub> 2, HPO<sub>4</sub> 1.8

1 gm molecule = 1,000 millimols    Millimols per litre  $\times$  valency  
= milliequivalents per litre = c c of 0.1 normal per 100 c c"

Relation of  $pH$  to  $C_H$ 

$pH$	$C_H$ Gm. of Ions II per litre.	$C_H \times 10^4$ (Gm. of Ions II per 100 million litres)	$C_H$
1.0	$1.00 \times 10^{-1}$	10,000,000	1.0 gm. per 10 litres
1.1	0.80	8,000,000	8.0 " " 100 "
1.2	0.63	6,300,000	6.3 " " " "
1.3	0.50	5,000,000	5.0 " " " "
1.4	0.40	4,000,000	4.0 " " " "
1.5	0.32	3,200,000	3.2 " " " "
1.6	0.25	2,500,000	2.5 " " " "
1.7	0.20	2,000,000	2.0 " " " "
1.8	0.16	1,600,000	1.6 " " " "
1.9	0.13	1,300,000	1.3 " " " "
2.0	$1.00 \times 10^{-2}$	1,000,000	1.0 " " " "
2.1	0.80	800,000	8.0 " " 1,000 "
2.2	0.63	630,000	6.3 " " " "
2.3	0.50	500,000	5.0 " " " "
2.4	0.40	400,000	4.0 " " " "
2.5	0.32	320,000	3.2 " " " "
2.6	0.25	250,000	2.5 " " " "
2.7	0.20	200,000	2.0 " " " "
2.8	0.16	160,000	1.6 " " " "
2.9	0.13	130,000	1.3 " " " "
3.0	$1.00 \times 10^{-3}$	100,000	1.0 " " " "
3.1	0.80	80,000	8.0 " " 10,000 "
3.2	0.63	63,000	6.3 " " " "
3.3	0.50	50,000	5.0 " " " "
3.4	0.40	40,000	4.0 " " " "
3.5	0.32	32,000	3.2 " " " "
3.6	0.25	25,000	2.5 " " " "
3.7	0.20	20,000	2.0 " " " "
3.8	0.16	16,000	1.6 " " " "
3.9	0.13	13,000	1.3 " " " "
4.0	$1.00 \times 10^{-4}$	10,000	1.0 " " " "
4.1	0.80	8,000	8.0 " " 100,000 "
4.2	0.63	6,300	6.3 " " " "
4.3	0.50	5,000	5.0 " " " "
4.4	0.40	4,000	4.0 " " " "
4.5	0.32	3,200	3.2 " " " "
4.6	0.25	2,500	2.5 " " " "
4.7	0.20	2,000	2.0 " " " "
4.8	0.16	1,600	1.6 " " " "
4.9	0.13	1,300	1.3 " " " "
5.0	$1.00 \times 10^{-5}$	1,000	1.0 " " " "
5.1	0.80	800	8.0 " " 1,000,000 "
5.2	0.63	630	6.3 " " " "
5.3	0.50	500	5.0 " " " "
5.4	0.40	400	4.0 " " " "
5.5	0.32	320	3.2 " " " "
5.6	0.25	250	2.5 " " " "
5.7	0.20	200	2.0 " " " "
5.8	0.16	160	1.6 " " " "
5.9	0.13	130	1.3 " " " "
6.0	$1.00 \times 10^{-6}$	100	1.0 " " " "
6.1	0.80	80	8.0 " " 10,000,000 "
6.2	0.63	63	6.3 " " " "
6.3	0.50	50	5.0 " " " "
6.4	0.40	40	4.0 " " " "
6.5	0.32	32	3.2 " " " "
6.6	0.25	25	2.5 " " " "
6.7	0.20	20	2.0 " " " "
6.8	0.16	16	1.6 " " " "
6.9	0.13	13	1.3 " " " "
7.0	$1.00 \times 10^{-7}$	10	1.0 " " " "
7.1	0.80	8	8.0 " " 100,000,000 "
7.2	0.63	6.3	6.3 " " " "
7.3	0.50	5.0	5.0 " " " "
7.4	0.40	4.0	4.0 " " " "
7.5	0.32	3.2	3.2 " " " "
7.6	0.25	2.5	2.5 " " " "
7.7	0.20	2.0	2.0 " " " "
7.8	0.16	1.6	1.6 " " " "
7.9	0.13	1.3	1.3 " " " "
8.0	$1.00 \times 10^{-8}$	1.0	1.0 " " " "

Relationship between the Three Thermometric Scales,  
Fahrenheit, Centigrade and Réaumur

To convert degrees F into degrees C deduct 32, multiply by 5, and divide by 9

To convert degrees C into degrees F, multiply by 9, divide by 5 and add 32

To convert degrees F into degrees R, deduct 32, multiply by 4, and divide by 9

To convert degrees R into degrees F multiply by 9, divide by 4, and add 32

Freezing point of water = 32° F      0° C and 0° R

Boiling point of water = 212° F      100° C and 80° R

°F	°C	°R	°F	°C	°R
212 0	100 0	80 0	95 0	35 0	28 0
200 0	93 3	74 7	94 0	34 4	27 6
150 0	65 6	52 4	92 0	33 3	26 7
112 0	44 4	35 5	90 0	32 2	25 8
110 0	43 3	34 7	88 0	31 1	24 9
108 0	42 2	33 8	86 0	30 0	24 0
106 0	41 1	32 9	84 0	28 9	23 1
105 0	40 6	32 4	82 0	27 8	22 2
104 0	40 0	32 0	80 0	26 7	21 3
103 0	39 4	31 6	78 0	25 6	20 4
102 0	38 9	31 1	76 0	24 4	19 6
101 5	38 6	30 9	74 0	23 3	18 7
101 0	38 3	30 7	72 0	22 2	17 8
100 5	38 1	30 4	70 0	21 1	16 9
100 0	37 8	30 2	68 0	20 0	16 0
99 5	37 5	30 0	66 0	18 9	15 1
99 0	37 2	29 8	64 0	17 8	14 2
98 4*	36 9	29 5	62 0	16 7	13 3
98 0	36 7	29 3	60 0	15 6	12 4
97 5	36 4	29 1	58 0	14 4	11 6
97 0	36 1	28 9	56 0	13 3	10 7
96 5	35 8	28 7	54 0	12 2	9 8
96 0	35 6	28 4	52 0	11 1	8 9
95 5	35 3	28 2	50 0	10 0	8 0

\* Temperature of human body

## Relationship of Imperial to Metric System

$\frac{1}{4}$ grain	=	16 milligrammes	
1 "	=	32 "	
1 "	=	65 "	= 0.065 gm.
10 grains	=	648 "	= 0.648 "
30 "	=	1.944 grammes	= 2 gm. (approx)
1 drachm (60 grains)	=	3.89 "	= 4 " "
1 ounce (480 grains)	=	31.1 "	= 31 " "
(Troy or Apothecaries')			
1 ounce (437.5 grains)	=	28.35 "	= 28 " "
(Avoirdupois)			
1 pound (7,000 grains)	=	453.6 "	= 454 " "
1 stone (14 lb)	=	6,350 "	= 6½ kgm. "
1 minim	=	0.06 c c	(0.059 c c.)
1 fluid drachm	=	3½ "	(3.6 " )
$\frac{1}{2}$ " ounce	=	14 "	(14.2 " )
1 " "	=	28 "	(28.4 " )
1 pint (20 oz)	=	568 "	(567.9 " )
1 gallon (8 pints)	=	4½ litres	(4542.0 " )
1 gramme	=	15½ grains	(15.4 grains)
100 grammes	=	3½ ounces	(Avoir)
1,000 " (1 kilogramme)	=	2½ lb.	(2.2 lb)
1 c c.	=	17 minims	(16.9 m)
100 "	=	3½ fluid oz	
1,000 " (1 litre)	=	35 " "	= 1½ pints.

Grammes per 100 c c	Grains per oz	Approximate grains per oz
1	4½	4½
2	8½	9
3	13½	13
4	17½	17½
5	21½	22
6	26½	26
7	30½	30½
8	35	35
9	39½	39½
10	43½	44

One drachm (by weight) of a dry powder to 1 oz. of water makes approximately a 12 per cent. solution (12.5 per cent.).

One drachm (by weight) of a dry powder to 1 pint of water makes approximately a  $\frac{1}{2}$  per cent. solution (0.63 per cent.).

One ounce (by weight) of a dry powder to a pint of water makes approximately a 5 per cent. solution.

1 inch = 2½ cm. (2.54 cm.)

1 foot = 30 cm. (30.48 cm.)

1 yard = 91 cm. (91.44 cm.)

1 mm. =  $\frac{1}{25}$  inch.

1 cm. =  $\frac{1}{25}$  inch (0.39 inch).

1 metre = 39 inches (39.37 inches).

## ATOMIC WEIGHTS

Name	Sym bol	Approxi mate	Inter national (1934)	Name	Sym bol	Approxi mate	Inter- national (1934)
Aluminium	Al	27	26.97	Molybdenum	Mo	96	96.0
Antimony	Sb	122	121.76	Nickel	Ni	59	58.69
Arsenic	As	75	74.91	Nitrogen	N	14	14.008
Barium	Ba	137	137.36	Osmium	Os	191	191.5
Bismuth	Bi	209	209.00	Oxygen	O	16	16.00
Boron	B	11	10.82	Palladium	Pd	107	106.7
Bromine	Br	80	79.916	Phosphorus	P	31	31.02
Cadmium	Cd	112	112.41	Platinum	Pt	195	195.23
Calcium	Ca	40	40.08	Potassium	K	39	39.096
Carbon	C	12	12.00	Radium	Ra	226	225.97
Chlorine	Cl	35.5	35.457	Radon	Rn	222	222.0
Chromium	Cr	52	52.01	Selenium	Se	79	78.96
Cobalt	Co	59	58.94	Silicon	Si	28	28.06
Copper	Cu	63.5	63.57	Silver	Ag	108	107.88
Fluorine	F	19	19.00	Sodium	Na	23	22.997
Gold	Au	197	197.2	Strontium	Sr	88	87.63
Hydrogen	H	1	1.008	Sulphur	S	32	32.06
Iodine	I	127	126.92	Tellurium	Te	127.5	127.61
Iron	Fe	56	55.84	Thallium	Tl	204	204.39
Lead	Pb	207	207.22	Tin	Sn	119	118.7
Lithium	Li	7	6.94	Tungsten	W	184	184.0
Magnesium	Mg	24	24.32	Uranium	U*	238	238.14
Manganese	Mn	55	54.93	Vanadium	V	51	50.95
Mercury	Hg	201	200.61	Zinc	Zn	65	65.38

## SPECIFIC GRAVITIES TABLES

(From Cole's *Practical Physiological Chemistry*)

## Sulphuric Acid

$\frac{Sp. Gr.}{15^{\circ}}_{4^{\circ}}$	Gm. of $H_2SO_4$ in 100 c c	$\frac{Sp. Gr.}{15^{\circ}}_{4^{\circ}}$	Gm. of $H_2SO_4$ in 100 c c
1 840	175 9	1 552	100 0
1 838	173 9	1 542	98 1
1 835	171 7	1 520	93 6
1 833	170 4	1 492	88 95
1 830	168 5	1 420	74 0
1 825	166 1	1 380	66 2
1 815	161 8	1 295	50 6
1 800	153 4	1 200	32 8

## Hydrochloric Acid

$\frac{Sp. Gr.}{15^{\circ}}_{4^{\circ}}$	Gm. of HCl in 100 c c	$\frac{Sp. Gr.}{15^{\circ}}_{4^{\circ}}$	Gm. of HCl in 100 c c
1 160	36 6	1 133	30
1 155	35 3	1 113	25
1 152	34 5	1 091	20
1 150	34 0	1 056	12
1 145	32 8	1 047	10
1 140	31 5	1 0375	8

## Sodium and Potassium Hydroxides

Sp. Gr $\frac{15^{\circ}}{4}$	Gm. of NaOH in 100 c.c.	Gm. of KOH in 100 c.c.
1 634	—	94 0
1 615	—	90 2
1 530	—	75 6
1 438	57 5	—
1 397	50 6	54 3
1 370	46 2	50 6
1 332	40 0	45 1
1 190	20 0	25 5

## Ammonia

Sp. Gr $\frac{15^{\circ}}{4}$	Gm. of $\text{NH}_3$ in 100 c.c.	Sp. Gr $\frac{15^{\circ}}{4}$	Gm. of $\text{NH}_3$ in 100 c.c.
880	31 0	896	26 6
882	30 83	898	26 05
884	30 14	900	25 5
886	29 46	902	24 94
888	28 86	906	23 83
890	28 26	910	22 74
892	27 70	920	20 01
894	27 15	926	18 42

## Alcohol

Sp. Gr. 15°56°.	Volume per cent.	Sp. Gr. 15°56°.	Volume per cent.
·79391	100	·83065	91
·79891	99	·83400	90
·80359	98	·86395	80
·80800	97	·87740	75
·81217	96	·89010	70
·81616	95	·90214	65
·81997	94	·91358	60
·82365	93	·92439	55
·82721	92	·93445	50



Tension of Aqueous Vapour. See p. 493.

### BOILING POINTS OF ORGANIC SOLVENTS

Substance	° C.
Ether . . . . .	34.2
Light petroleum (petroleum ether) . . . . .	40 to 90
Carbon disulphide . . . . .	46.2
Acetone . . . . .	56.5
Chloroform . . . . .	61 to 63
Methyl alcohol . . . . .	66
Ethyl acetate . . . . .	77
Ethyl alcohol . . . . .	78
Benzene (benzol) . . . . .	80
Butyl alcohol, secondary . . . . .	99.8
<i>iso</i> Butyl alcohol . . . . .	106
Toluene (toluol) . . . . .	111
Butyl alcohol, normal . . . . .	117
Acetic acid, glacial . . . . .	119
Amyl alcohol . . . . .	130
Xylenes (xylols) . . . . .	137 to 142
Caprylic alcohol . . . . .	179.5

### Calculation of the Calorific Value of a Diet

1 gramme of carbohydrate yields 4.1 Calories

1	"	protein	"	4.1	"
1	"	fat	"	9.3	"
1	"	alcohol	"	7.0	"
1 c.c.	"	"	"	5.6	"

For general work it is convenient to regard 1 gm. of carbohydrate or of protein as yielding 4 Cals., and 1 gm. of fat, 9 Cals.

## LOGARITHMS

											Proportional parts									
	0	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9	
10	0000	0043	0086	0128	0170	0212	0253	0294	0334	0374	4	8	12	17	21	25	29	33	37	
11	0414	0453	0492	0531	0569	0607	0645	0682	0719	0755	4	8	11	15	19	23	26	30	34	
12	0792	0828	0864	0899	0934	0969	1001	1038	1072	1106	3	7	10	14	17	21	24	28	31	
13	1139	1173	1206	1239	1271	1303	1335	1367	1399	1430	3	6	10	13	16	19	23	26	29	
14	1461	1492	1523	1553	1584	1614	1644	1673	1703	1732	3	6	9	12	15	18	21	24	27	
15	1761	1790	1818	1847	1875	1903	1931	1959	1987	2014	3	6	8	11	14	17	20	22	25	
16	2041	2068	2095	2122	2148	2175	2201	2227	2253	2279	3	5	8	11	13	16	18	21	24	
17	2304	2330	2355	2380	2405	2430	2455	2480	2504	2529	2	5	7	10	12	15	17	20	22	
18	2553	2577	2601	2625	2648	2672	2695	2718	2742	2765	2	5	7	9	12	14	16	19	21	
19	2788	2810	2833	2856	2878	2900	2923	2945	2967	2989	2	4	7	9	11	13	16	18	20	
20	3010	3032	3054	3075	3096	3118	3139	3160	3181	3201	2	4	6	8	11	13	15	17	19	
21	3222	3243	3263	3284	3304	3324	3345	3365	3385	3404	2	4	6	8	10	12	14	16	18	
22	3424	3444	3464	3483	3502	3522	3541	3560	3579	3598	2	4	6	8	10	12	14	15	17	
23	3617	3636	3655	3674	3692	3711	3729	3747	3766	3784	2	4	6	7	9	11	13	15	17	
24	3802	3820	3838	3856	3874	3892	3909	3927	3945	3962	2	4	5	7	9	11	12	14	16	
25	3979	3997	4014	4031	4048	4065	4082	4099	4116	4133	2	3	5	7	9	10	12	14	15	
26	4150	4166	4183	4200	4216	4232	4249	4265	4281	4298	2	3	5	7	8	10	11	13	15	
27	4314	4330	4346	4362	4378	4393	4409	4425	4440	4456	2	3	5	6	8	9	11	13	14	
28	4472	4487	4502	4518	4533	4548	4564	4579	4594	4609	2	3	5	6	8	9	11	12	14	
29	4624	4639	4654	4669	4683	4698	4713	4728	4742	4757	1	3	4	6	7	9	10	12	13	
30	4771	4786	4800	4814	4829	4843	4857	4871	4886	4900	1	3	4	6	7	9	10	11	13	
31	4914	4928	4942	4955	4969	4983	4997	5011	5024	5038	1	3	4	6	7	8	10	11	12	
32	5051	5065	5079	5092	5105	5119	5132	5145	5159	5172	1	3	4	5	7	8	9	11	12	
33	5185	5198	5211	5224	5237	5250	5263	5276	5289	5302	1	3	4	5	6	8	9	10	12	
34	5315	5328	5340	5353	5366	5378	5391	5403	5416	5428	1	3	4	5	6	8	9	10	11	
35	5441	5453	5465	5478	5490	5502	5514	5527	5539	5551	1	2	4	5	6	7	9	10	11	
36	5563	5575	5587	5599	5611	5623	5635	5647	5658	5670	1	2	4	5	6	7	8	10	11	
37	5682	5694	5705	5717	5729	5740	5752	5763	5775	5786	1	2	3	5	6	7	8	9	10	
38	5798	5809	5821	5832	5843	5855	5866	5877	5888	5899	1	2	3	5	6	7	8	9	10	
39	5911	5922	5933	5944	5955	5966	5977	5988	5999	6010	1	2	3	4	5	7	8	9	10	
40	6021	6031	6042	6053	6064	6075	6085	6096	6107	6117	1	2	3	4	5	6	8	9	10	
41	6128	6138	6149	6160	6170	6180	6191	6201	6212	6222	1	2	3	4	5	6	7	8	9	
42	6232	6243	6253	6263	6274	6284	6294	6304	6314	6325	1	2	3	4	5	6	7	8	9	
43	6335	6345	6355	6365	6375	6385	6395	6405	6415	6425	1	2	3	4	5	6	7	8	9	
44	6435	6444	6454	6464	6474	6484	6493	6503	6513	6522	1	2	3	4	5	6	7	8	9	
45	6532	6542	6551	6561	6571	6580	6590	6599	6609	6618	1	2	3	4	5	6	7	8	9	
46	6628	6637	6646	6656	6665	6675	6684	6693	6702	6712	1	2	3	4	5	6	7	7	8	
47	6721	6730	6739	6749	6758	6767	6776	6785	6794	6803	1	2	3	4	5	5	6	7	8	
48	6812	6821	6830	6839	6848	6857	6866	6875	6884	6893	1	2	3	4	4	5	6	7	8	
49	6902	6911	6920	6928	6937	6946	6955	6964	6972	6981	1	2	3	4	4	5	6	7	8	
50	6990	6998	7007	7016	7024	7033	7042	7050	7059	7067	1	2	3	3	4	5	6	7	8	
51	7076	7084	7093	7101	7110	7118	7126	7135	7143	7152	1	2	3	3	4	5	6	7	8	
52	7160	7168	7177	7185	7193	7202	7210	7218	7226	7235	1	2	2	3	4	5	6	7	7	
53	7243	7251	7259	7267	7275	7284	7292	7300	7308	7316	1	2	2	3	4	5	6	6	7	
54	7324	7332	7340	7348	7356	7364	7372	7380	7388	7396	1	2	2	3	4	5	6	6	7	
	0	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9	

## LOGARITHMS

											Proportional parts.									
	0	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9	
55	7404	7412	7419	7427	7435	7443	7451	7459	7466	7474	1	2	2	3	4	5	5	6	7	
56	7482	7490	7497	7505	7513	7520	7528	7536	7543	7551	1	2	2	3	4	5	5	6	7	
57	7559	7566	7574	7582	7589	7597	7604	7612	7619	7627	1	2	2	3	4	5	5	6	7	
58	7634	7642	7649	7657	7664	7672	7679	7686	7694	7701	1	1	2	3	4	4	5	6	7	
59	7709	7716	7723	7731	7738	7745	7752	7760	7767	7774	1	1	2	3	4	4	5	6	7	
60	7782	7789	7796	7803	7810	7818	7825	7832	7839	7846	1	1	2	3	4	4	5	6	6	
61	7853	7860	7868	7875	7882	7889	7896	7903	7910	7917	1	1	2	3	4	4	5	6	6	
62	7924	7931	7938	7945	7952	7959	7966	7973	7980	7987	1	1	2	3	3	4	5	6	6	
63	7993	8000	8007	8014	8021	8028	8035	8041	8048	8055	1	1	2	3	3	4	5	5	6	
64	8062	8069	8075	8082	8089	8096	8102	8109	8116	8122	1	1	2	3	3	4	5	5	6	
65	8129	8136	8142	8149	8156	8162	8169	8176	8182	8189	1	1	2	3	3	4	5	5	6	
66	8195	8202	8209	8215	8222	8228	8235	8241	8248	8254	1	1	2	3	3	4	5	5	6	
67	8261	8267	8274	8280	8287	8293	8299	8306	8312	8319	1	1	2	3	3	4	5	5	6	
68	8325	8331	8338	8344	8351	8357	8363	8370	8376	8382	1	1	2	3	3	4	4	5	6	
69	8388	8395	8401	8407	8414	8420	8426	8432	8439	8445	1	1	2	2	3	4	4	5	6	
70	8451	8457	8463	8470	8476	8482	8488	8494	8500	8506	1	1	2	2	3	4	4	5	6	
71	8513	8519	8525	8531	8537	8543	8549	8555	8561	8567	1	1	2	2	3	4	4	5	5	
72	8573	8579	8585	8591	8597	8603	8609	8615	8621	8627	1	1	2	2	3	4	4	5	5	
73	8633	8639	8645	8651	8657	8663	8669	8675	8681	8686	1	1	2	2	3	4	4	5	5	
74	8692	8698	8704	8710	8716	8722	8727	8733	8739	8745	1	1	2	2	3	4	4	5	5	
75	8751	8756	8762	8768	8774	8779	8785	8791	8797	8802	1	1	2	2	3	3	4	5	5	
76	8808	8814	8820	8825	8831	8837	8842	8848	8854	8859	1	1	2	2	3	3	4	5	5	
77	8865	8871	8876	8882	8887	8893	8899	8904	8910	8915	1	1	2	2	3	3	4	4	5	
78	8921	8927	8932	8938	8943	8949	8954	8960	8965	8971	1	1	2	2	3	3	4	4	5	
79	8976	8982	8987	8993	8998	9004	9009	9015	9020	9025	1	1	2	2	3	3	4	4	5	
80	9031	9036	9042	9047	9053	9058	9063	9069	9074	9079	1	1	2	2	3	3	4	4	5	
81	9085	9090	9096	9101	9106	9112	9117	9122	9128	9133	1	1	2	2	3	3	4	4	5	
82	9138	9143	9149	9154	9159	9165	9170	9175	9180	9186	1	1	2	2	3	3	4	4	5	
83	9191	9196	9201	9206	9212	9217	9222	9227	9232	9238	1	1	2	2	3	3	4	4	5	
84	9243	9248	9253	9258	9263	9269	9274	9279	9284	9289	1	1	2	2	3	3	4	4	5	
85	9294	9299	9304	9309	9315	9320	9325	9330	9335	9340	1	1	2	2	3	3	4	4	5	
86	9345	9350	9355	9360	9365	9370	9375	9380	9385	9390	1	1	2	2	3	3	4	4	5	
87	9395	9400	9405	9410	9415	9420	9425	9430	9435	9440	0	1	1	2	2	3	3	4	4	
88	9445	9450	9455	9460	9465	9469	9474	9479	9484	9489	0	1	1	2	2	3	3	4	4	
89	9494	9499	9504	9509	9513	9518	9523	9528	9533	9538	0	1	1	2	2	3	3	4	4	
90	9542	9547	9552	9557	9562	9566	9571	9576	9581	9586	0	1	1	2	2	3	3	4	4	
91	9590	9595	9600	9605	9609	9614	9619	9624	9628	9633	0	1	1	2	2	3	3	4	4	
92	9638	9643	9647	9652	9657	9661	9666	9671	9675	9680	0	1	1	2	2	3	3	4	4	
93	9685	9689	9694	9699	9703	9708	9713	9717	9722	9727	0	1	1	2	2	3	3	4	4	
94	9731	9736	9741	9745	9750	9754	9759	9763	9768	9773	0	1	1	2	2	3	3	4	4	
95	9777	9782	9786	9791	9795	9800	9805	9809	9814	9818	0	1	1	2	2	3	3	4	4	
96	9823	9827	9832	9836	9841	9845	9850	9854	9859	9863	0	1	1	2	2	3	3	4	4	
97	9868	9872	9877	9881	9886	9890	9894	9899	9903	9908	0	1	1	2	2	3	3	4	4	
98	9912	9917	9921	9926	9930	9934	9939	9943	9948	9952	0	1	1	2	2	3	3	4	4	
99	9956	9961	9965	9969	9974	9978	9983	9987	9991	9996	0	1	1	2	2	3	3	4	4	
	0	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9	

## ANTI-LOGARITHMS

											Proportional parts.									
	0	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9	
00	1000	1002	1005	1007	1009	1012	1014	1016	1019	1021	0	0	1	1	1	1	2	2	2	
01	1023	1026	1028	1030	1033	1035	1038	1040	1042	1045	0	0	1	1	1	1	2	2	2	
02	1047	1050	1052	1054	1057	1059	1062	1064	1067	1069	0	0	1	1	1	1	2	2	2	
03	1072	1074	1076	1079	1081	1084	1086	1089	1091	1094	0	0	1	1	1	1	2	2	2	
04	1096	1099	1102	1104	1107	1109	1112	1114	1117	1119	0	1	1	1	1	2	2	2	2	
05	1122	1125	1127	1130	1132	1135	1138	1140	1143	1146	0	1	1	1	1	2	2	2	2	
06	1148	1151	1153	1156	1159	1161	1164	1167	1169	1172	0	1	1	1	1	2	2	2	2	
07	1175	1178	1180	1183	1186	1189	1191	1194	1197	1199	0	1	1	1	1	2	2	2	2	
08	1202	1205	1208	1211	1213	1216	1219	1222	1225	1227	0	1	1	1	1	2	2	2	3	
09	1230	1233	1236	1239	1242	1245	1247	1250	1253	1256	0	1	1	1	1	2	2	2	3	
10	1259	1262	1265	1268	1271	1274	1276	1279	1282	1285	0	1	1	1	1	2	2	2	3	
11	1288	1291	1294	1297	1300	1303	1306	1309	1312	1315	0	1	1	1	2	2	2	2	3	
12	1318	1321	1324	1327	1330	1334	1337	1340	1343	1346	0	1	1	1	2	2	2	2	3	
13	1349	1352	1355	1358	1361	1365	1368	1371	1374	1377	0	1	1	1	2	2	2	2	3	
14	1380	1384	1387	1390	1393	1396	1400	1403	1406	1409	0	1	1	1	2	2	2	2	3	
15	1413	1416	1419	1422	1426	1429	1432	1435	1439	1442	0	1	1	1	2	2	2	2	3	
16	1445	1449	1452	1455	1459	1462	1466	1469	1472	1476	0	1	1	1	2	2	2	2	3	
17	1479	1483	1486	1489	1493	1496	1500	1503	1507	1510	0	1	1	1	2	2	2	2	3	
18	1514	1517	1521	1524	1528	1531	1535	1538	1542	1545	0	1	1	1	2	2	2	2	3	
19	1549	1552	1556	1560	1563	1567	1570	1574	1578	1581	0	1	1	1	2	2	2	2	3	
20	1585	1589	1592	1596	1600	1603	1607	1611	1614	1618	0	1	1	1	2	2	2	2	3	
21	1622	1626	1629	1633	1637	1641	1644	1648	1652	1656	0	1	1	2	2	2	2	2	3	
22	1660	1663	1667	1671	1675	1679	1683	1687	1690	1694	0	1	1	2	2	2	2	2	3	
23	1698	1702	1706	1710	1714	1718	1722	1726	1730	1734	0	1	1	2	2	2	2	2	3	
24	1738	1742	1746	1750	1754	1758	1762	1766	1770	1774	0	1	1	2	2	2	2	2	3	
25	1778	1782	1786	1791	1795	1799	1803	1807	1811	1816	0	1	1	2	2	2	2	2	3	
26	1820	1824	1828	1832	1837	1841	1845	1849	1854	1858	0	1	1	2	2	2	2	2	3	
27	1862	1866	1871	1875	1879	1884	1888	1892	1897	1901	0	1	1	2	2	2	2	2	3	
28	1905	1910	1914	1919	1923	1928	1932	1936	1941	1945	0	1	1	2	2	2	2	2	3	
29	1950	1954	1959	1963	1968	1972	1977	1982	1986	1991	0	1	1	2	2	2	2	2	3	
30	1995	2000	2004	2009	2014	2018	2023	2028	2032	2037	0	1	1	2	2	2	2	2	3	
31	2042	2046	2051	2056	2061	2065	2070	2075	2080	2084	0	1	1	2	2	2	2	2	3	
32	2089	2094	2099	2104	2109	2113	2118	2123	2128	2133	0	1	1	2	2	2	2	2	3	
33	2138	2143	2148	2153	2158	2163	2168	2173	2178	2183	0	1	1	2	2	2	2	2	3	
34	2188	2193	2198	2203	2208	2213	2218	2223	2228	2234	0	1	1	2	2	2	2	2	3	
35	2239	2244	2249	2254	2259	2265	2270	2275	2280	2286	0	1	1	2	2	2	2	2	3	
36	2291	2296	2301	2307	2312	2317	2323	2328	2333	2339	0	1	1	2	2	2	2	2	3	
37	2344	2350	2355	2360	2366	2371	2377	2382	2388	2393	0	1	1	2	2	2	2	2	3	
38	2399	2404	2410	2415	2421	2427	2432	2438	2443	2449	0	1	1	2	2	2	2	2	3	
39	2455	2460	2466	2472	2477	2483	2489	2495	2500	2506	1	1	2	2	2	2	2	2	3	
40	2512	2518	2523	2529	2535	2541	2547	2553	2559	2564	1	1	2	2	2	2	2	2	3	
41	2570	2576	2582	2588	2594	2600	2606	2612	2618	2624	1	1	2	2	2	2	2	2	3	
42	2630	2636	2642	2649	2655	2661	2667	2673	2679	2685	1	1	2	2	2	2	2	2	3	
43	2692	2698	2704	2710	2716	2723	2729	2735	2742	2748	1	1	2	2	2	2	2	2	3	
44	2754	2761	2767	2773	2780	2786	2793	2799	2805	2812	1	1	2	2	2	2	2	2	3	
45	2818	2825	2831	2838	2844	2851	2858	2864	2871	2877	1	1	2	2	2	2	2	2	3	
46	2884	2891	2897	2904	2911	2917	2924	2931	2938	2944	1	1	2	2	2	2	2	2	3	
47	2951	2958	2965	2972	2979	2985	2992	2999	3006	3013	1	1	2	2	2	2	2	2	3	
48	3020	3027	3034	3041	3048	3055	3062	3069	3076	3083	1	1	2	2	2	2	2	2	3	
49	3090	3097	3105	3112	3119	3126	3133	3141	3148	3155	1	1	2	2	2	2	2	2	3	
	0	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9	

## ANTI-LOGARITHMS

											Proportional parts.									
	0	1	2	3	4	5	6	7	.8	9	1	2	3	4	5	6	7	8	9	
50	3162	3170	3177	3184	3192	3199	3206	3214	3221	3228	1	1	2	3	4	4	5	6	7	
51	3236	3243	3251	3258	3266	3273	3281	3289	3296	3304	1	2	2	3	4	5	5	6	7	
52	3311	3319	3327	3334	3342	3350	3357	3365	3373	3381	1	2	2	3	4	5	5	6	7	
53	3388	3396	3404	3412	3420	3428	3436	3443	3451	3459	1	2	2	3	4	5	6	6	7	
54	3467	3475	3483	3491	3499	3508	3516	3524	3532	3540	1	2	2	3	4	5	6	6	7	
55	3548	3556	3565	3573	3581	3589	3597	3606	3614	3622	1	2	2	3	4	5	6	7	8	
56	3631	3639	3648	3656	3664	3673	3681	3690	3698	3707	1	2	3	3	4	5	6	7	8	
57	3715	3724	3733	3741	3750	3758	3767	3776	3784	3793	1	2	3	3	4	5	6	7	8	
58	3802	3811	3819	3828	3837	3846	3855	3864	3873	3882	1	2	3	4	4	5	6	7	8	
59	3890	3899	3908	3917	3926	3935	3945	3954	3963	3972	1	2	3	4	5	5	6	7	8	
60	3981	3990	3999	4009	4018	4027	4036	4046	4055	4064	1	2	3	4	5	6	6	7	8	
61	4074	4083	4093	4102	4111	4121	4130	4140	4150	4159	1	2	3	4	5	6	7	8	9	
62	4169	4178	4188	4198	4207	4217	4227	4236	4246	4256	1	2	3	4	5	6	7	8	9	
63	4266	4276	4285	4295	4305	4315	4325	4335	4345	4355	1	2	3	4	5	6	7	8	9	
64	4365	4375	4385	4395	4406	4416	4426	4436	4446	4457	1	2	3	4	5	6	7	8	9	
65	4467	4477	4487	4498	4508	4519	4529	4539	4550	4560	1	2	3	4	5	6	7	8	9	
66	4571	4581	4592	4603	4613	4624	4634	4645	4656	4667	1	2	3	4	5	6	7	9	10	
67	4677	4688	4699	4710	4721	4732	4742	4753	4764	4775	1	2	3	4	5	7	8	9	10	
68	4786	4797	4808	4819	4831	4842	4853	4864	4875	4887	1	2	3	4	6	7	8	9	10	
69	4898	4909	4920	4932	4943	4955	4966	4977	4989	5000	1	2	3	5	6	7	8	9	10	
70	5012	5023	5035	5047	5058	5070	5082	5093	5105	5117	1	2	4	5	6	7	8	9	11	
71	5129	5140	5152	5164	5176	5188	5200	5212	5224	5236	1	2	4	5	6	7	8	10	11	
72	5248	5260	5272	5284	5297	5309	5321	5333	5346	5358	1	2	4	5	6	7	9	10	11	
73	5370	5383	5395	5408	5420	5433	5445	5458	5470	5483	1	3	4	5	6	8	9	10	11	
74	5495	5508	5521	5534	5546	5559	5572	5585	5598	5610	1	3	4	5	6	8	9	10	12	
75	5623	5636	5649	5662	5675	5689	5702	5715	5728	5741	1	3	4	5	7	8	9	10	12	
76	5754	5768	5781	5794	5808	5821	5834	5848	5861	5875	1	3	4	5	7	8	9	11	12	
77	5888	5902	5916	5929	5943	5957	5970	5984	5998	6012	1	3	4	5	7	8	10	11	12	
78	6026	6039	6053	6067	6081	6095	6109	6124	6138	6152	1	3	4	6	7	8	10	11	13	
79	6166	6180	6194	6209	6223	6237	6252	6266	6281	6295	1	3	4	6	7	9	10	11	13	
80	6310	6324	6339	6353	6368	6383	6397	6412	6427	6442	1	3	4	6	7	9	10	12	13	
81	6457	6471	6486	6501	6516	6531	6546	6561	6577	6592	2	3	5	6	8	9	11	12	14	
82	6607	6622	6637	6653	6668	6683	6699	6714	6730	6745	2	3	5	6	8	9	11	12	14	
83	6761	6776	6792	6808	6823	6839	6855	6871	6887	6902	2	3	5	6	8	9	11	13	15	
84	6918	6934	6950	6966	6982	6998	7015	7031	7047	7063	2	3	5	6	8	10	11	13	15	
85	7079	7096	7112	7129	7145	7161	7178	7194	7211	7228	2	3	5	7	8	10	12	13	15	
86	7244	7261	7278	7295	7311	7328	7345	7362	7379	7396	2	3	5	7	8	10	12	13	15	
87	7413	7430	7447	7464	7482	7499	7516	7534	7551	7568	2	3	5	7	9	10	12	14	16	
88	7586	7603	7621	7638	7656	7674	7691	7709	7727	7745	2	4	5	7	9	11	12	14	16	
89	7762	7780	7798	7816	7834	7852	7870	7889	7907	7925	2	4	5	7	9	11	13	14	16	
90	7943	7962	7980	7998	8017	8035	8054	8072	8091	8110	2	4	6	7	9	11	13	15	17	
91	8128	8147	8166	8185	8204	8222	8241	8260	8279	8299	2	4	6	8	9	11	13	15	17	
92	8318	8337	8356	8375	8395	8414	8433	8453	8472	8492	2	4	6	8	10	12	14	15	17	
93	8511	8531	8551	8570	8590	8610	8630	8650	8670	8690	2	4	6	8	10	12	14	16	18	
94	8710	8730	8750	8770	8790	8810	8831	8851	8872	8892	2	4	6	8	10	12	14	16	18	
95	8913	8933	8954	8974	8995	9016	9036	9057	9078	9099	2	4	6	8	10	12	15	17	19	
96	9120	9141	9162	9183	9204	9226	9247	9268	9290	9311	2	4	6	8	11	13	15	17	19	
97	9333	9354	9376	9397	9419	9441	9462	9484	9506	9528	2	4	7	9	11	13	15	17	20	
98	9550	9572	9594	9616	9638	9661	9683	9705	9727	9750	2	4	7	9	11	13	16	18	20	
99	9772	9795	9817	9840	9863	9886	9908	9931	9954	9977	2	5	7	9	11	14	16	18	20	
	0	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9	

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